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Coláiste na hOllscoile Corcaigh

Effect of dairy based food ingredients on the gut microbiota of older consumers

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by

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Table of Contents

List of Figures

List of Supplementary Figures

List of Tables

List of Supplementary Tables

List of Abbreviations

AA	amino acids
AAD	antibiotic associated diarrhoea
Abx	antibiotics
AG	arabinogalactan
α-GOS	α-galactooligosaccharides
α-lac	alpha-lactalbumin
BCAA	branched chain amino acids
BMO	bovine milk oligosaccharides
CD	Crohn's disease
CDI	*Clostridium difficile* infection
CDV	cardiovascular disease
CLA	conjugated linoleic acid
CMP	caseinomacropeptide
COM	community
CONS	coagulase negative staphylococci
CRC	colorectal cancer
CRP	C-reactive protein
C-section	caesarean section
EAA	essential amino acids
eCB	endocannabinoid system
FGID	functional gastrointestinal disorders
FISH	fluorescent *in situ* hybridisation
FMT	faecal material transplantation

FOS	fructo-oligosaccharides
FSR	fraction synthetic rates
GABA	γ-aminobutyric acid
Gal	galactose
GBA	gut-brain axis
gDNA	genomic DNA
GF	germ free
GIT	gastrointestinal tract
GlcNAc	*N*-acetylglucosamine
GMP	glycomacropeptide
GOS	galacto-oligosaccharides
HMA	human microbiota associated
HMO	human milk oligosaccharides
HPA	hypothalamic-pituitary-adrenal
HPLC	high performance liquid chromatography
IBD	inflammatory bowel disease
IBS	irritable bowel syndrome
IBS-C	constipation-predominant
IBS-D	diarrhoea-predominant
IS	immune system
κ-CN	kappa casein
LC-AX	long-chain arabinoxylan
LDL-C	low-density lipoprotein cholesterol
LNP	lactose non-persistent
LOS	late onset sepsis

LPS	lipopolysaccharides
LS	longstay
MAMPs	microbial-associated molecular patterns
MCC	microbiome culture collection
MetS	metabolic syndrome
MFGM	milk fat globule membranes
MPB	muscle protein breakdown
MPS	muscle protein synthesis
mTOR	mechanistic target of rapamycin
NAFLD	non-alcoholic fatty liver disease
NASH	non-alcoholic steatohepatitis
NEC	necrotising colitis
NeuAc	*N*-acetylneuraminic acid
NeuGc	*N*-glycolylneuraminic acid
NF-κB	nuclear factor κB
NGP	next-generation probiotics
NSP	non-starch polysaccharides
OS	oligosaccharides
OTU	operational taxonomic unit
PAG	phenylacetylglutamine
PBS	phosphate buffer saline
PCoA	principal coordinates analysis
PCS	p-cresol sulphate
Phe	phenylalanine
PKU	phenylketonuria

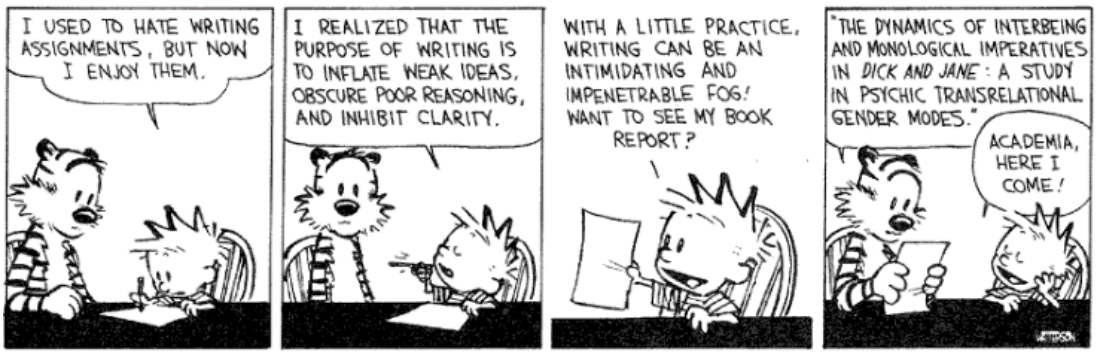
PUFA	polyunsaturated fatty acids
RS	resistant starch
SCFA	short chain fatty acids
SFA	saturated fatty acids
SIBO	small intestinal bacterial overgrowth
sWPI	sweet whey protein
T2D	type 2 diabetes
tGMP	semi-purified GMP
TNF-α	tumour necrosis factor α
Trp	tryptophan
Tyr	tyrosine
UC	ulcerative colitis
WHO	world health organisation
WT	wild type
XOS	xylo-oligosaccharides

Declaration

This is to certify that the work I am submitting is my own and has not been submitted for another degree, either at University College Cork or elsewhere. All extrenal references and sources are clearly acknowledged and identified within the contents. I have read and understood the regulations of University College Cork consnrinig plagiarism

Signed:

Alexandra Ntemiri, January 2019



Calvin and Hobbs, by Bill Watterson

Abstract

The proportion of the world population aged over 60 yrs is steadily increasing. Non-communicable disease and cognitive decline are prevalent in the elderly population which present a significant burden for national health care systems and economies. Malnutrition is prevalent in many longterm care unit-residing older people and importantly, it is also common among community-living elderly people. The deficiency in the aged population in certain nutrients, such as vitamins and proteins, may be associated with dementia and loss of bone and muscular health. Importantly, frailty that is accompanied by sarcopenia (i.e. deregulation of muscular homeostasis) is prevalent among older adults and may gradually lead to functional disability. Many studies have shown the importance of dietary management of aged-related conditions.

In the last decade, our knowledge of the importance of the gut microbiota in health and disease has significantly increased. Importantly, shifts in the composition and diversity of the gut microbiota of older people compared to healthy young adults are being identified and associated with ageing and age-related conditions, such as frailty. Similarly to other conditions for which gut microbiota modulation may lead to significant improvement of health, microbiota modulation may be a novel strategy for preventing or ameliorating ageing. This proposed gut microbiota modulation could be achieved through the use of prebiotics, probiotics or live bacteriotherapy among others. Importantly, diet can be a means of general (not targeted) gut microbiota modulation due to its documented significance in the shaping of the gut microbiota and to the fact that common dietary components may be a source of novel prebiotics.

In the ElderFood project, we investigated the prebiotic potential of milk and milk-derived components to modulate the gut microbiota of older subjects. Using *in vitro* and *in vivo* colon models, we generated an in-depth insight into the effect of glycomacropeptide (GMP), a milk-derived peptide that contains mucin-type glycans, and milk (whole and lactose free) on the elderly gut microbiota. We also performed exploratory work for the investigation of fibre and polyphenol-rich foods on the elderly gut microbiota and on artificial bacterial communities. The use of artificial bacterial communities can increase our understanding of the microbiota dynamics and how microbiota members respond to diet.

Chapter 1

Literature Review

1.1 From birth to old age: Factors that shape the human gut microbiome

Chapter 1 was published as a book chapter:

Ntemiri, A., Stanton, C., Ross, R. P., O'Toole, P. W. From birth to old age: Factors that shape the human gut microbiome. In "The Human Microbiome Handbook" Tetro JA, Allen-Vercoe E, eds. Lancaster, PA, **2016**. pp 35-72.

1.1.1 Abstract

The term microbiome generally refers to the microbiota and the collective genetic information those organisms carry. This genetic information supplements the genomic and metabolic potential of the host. A common approach to analysing the microbiome is to monitor compositional and functional changes in the microbiota and then attempting to correlate those changes with health or disease symptoms. This is usually a prelude to attempting to identify mechanisms and effectors.

Largely based on culture-independent techniques, the core composition of the intestinal microbiota has recently been identified. The same phyla, taxa and genera are generally found in the microbiota of the gastrointestinal tract of humans irrespective of the condition of the subjects under testing, that is, healthy or non-healthy young or older subjects. Thus, selective pressure and co-evolution between the host and the intestinal commensals have apparently established a functionally stable microbiota composition in the gut. Differences in the relative abundance of bacterial subgroups in the gut microbiota, i.e. dysbiosis, indicate changes in the health status of the host, or major environmental perturbations such as diet or antibiotics.

The changes in the microbiota from early colonisation events to those that occur during ageing are summarised here. Habitual diet is postulated to be the most determinative factor in shaping the human gut microbiome. “Westernised diet” has been identified as a major link between nutrient and gut microbiota signalling, inflammation and metabolic disease. Dysbiosis also characterises functional disorders of the gastrointestinal track like irritable bowel syndrome and inflammatory bowel disease. The microbiota of the gastrointestinal tract of older

66 subjects differs from that of young healthy adults. A key aspect in future research is
67 to identify the exact molecular mechanisms that link dysbiosis and disease in order
68 to develop therapeutic strategies based on microbiota manipulation or administration
69 of microbial products.

1.1.2 Introduction

The term microbiome refers to the microbiota collection of microorganisms or in a particular site and more specifically to the collective genetic information of those organisms. Investigation of the association of gut microbiota to phenotypes of health or disease is a major feature of contemporary microbiome research (Cho and Blaser, 2012). Culture-independent techniques and massive parallel next-generation sequencing has generated data that enabled the profiling of the gut microbiota of various healthy and disease cohorts ranging from infancy to the extremities of life span as discussed in following chapters.

The phyla Firmicutes and Bacteroidetes are the most predominant in the healthy adult gut microbiota and around 90 % of the bacterial groups found in the intestine typically belong to these phyla, whereas Actinobacteria, Proteobacteria and Verrucomicrobia comprise up to around 2 %, 1 % and 0.1 % respectively of the intestinal microbiota composition (Hold *et al*., 2002; Wang *et al*., 2003; Eckburg *et al*., 2005; Rajilic-Stojanovic *et al*., 2009; Tap *et al*., 2009; Qin *et al*., 2010). Dominant bacterial groups in the gut microbiota belong to the genera *Faecalibacterium*, *Ruminococcus*, *Eubacterium*, *Dorea*, *Bacteroides*, *Alistipes* and *Bifidobacterium* (Tap *et al*., 2009). Eckburg *et al* (2005) also identified substantial representation of butyrate–producing Firmicutes belonging mostly to clostridia (clusters IV, XIVa) and of *Bacteroides thetaiotaomicron* in the intestinal microbiota, both subgroups known for exerting beneficial effects upon the host (Wrzosek *et al*., 2013).

The gut microbiota composition as described above is based on studies on the intestinal microbiota of westernised urban cohorts. There are exceptions to this

general picture of the composition of the gut microbiota. A recent study on the gut microbiota of healthy hunter-gatherers in Hadza of Tanzania revealed the absence of bifidobacteria and other major compositional differences compared to a European urban cohort (Schnorr *et al*, 2014). Strong selective pressure during co-evolution of commensal gut bacteria and the mammalian host (Ley *et al*., 2009) apparently resulted in the establishment of symbiotic relationships that allow the host to extract the maximum energy from the available habitual diet sources. It seems that the gut microbiota of the Hadza cohort is adapted to a certain diet which is significantly different to the westernised urban diet.

Host genetics seems to play an important role in shaping the microbiome profile (approximately 2% contribution of genetics; Rothschild *et al*., 2018) and potentially explains some of the inter-individual variations in predominant species, subgroups and phylotypes (Eckburg *et al.*, 2005; Benson *et al*., 2010). However, a more determinative role in the composition of the gut microbiota has been attributed to habitual diet compared to host-genetics (Zhang *et al*., 2010). Rothschild *et al*. (2018) reported that environmental factors such as diet and lifestyle contribute over 20% to the microbiota variability observed between individuals. Recently, bacterial groups have been identified that are stably either absent or abundant over time in most individuals (based on a cohort of a thousand western adults) without being affected by short-term diet intervention and which correlated to health status such as overweight and ageing (Lahti *et al*., 2014).

It is becoming accepted that there is a functional redundancy in the gut microbiota (Mahowald *et al*., 2009; Lozupone *et al*., 2012) which explains how variant bacterial groups can result in the same functional core of the human gut microbiome

(Turnbaugh *et al*., 2009; Qin *et al*., 2010) Lessons from microbial ecology support the notion that bacterial community structure is rather explained from the point of functional genes than by species composition (Burke *et al*., 2011).

The gut microbiome shifts that occur throughout the lifespan and the factors driving these changes are reviewed here, including the early colonisation events of the gastrointestinal track and the changes observed during ageing. The important role of diet in modulating the intestinal microbiota is also discussed, summarising the existing evidence indicating that diet can underpin metabolic disorders and inflammation via the microbiota. Furthermore, we present the existing evidence linking gut microbiota and exercise. Finally, dysbiosis associated with antibiotic intake and functional disorders of the gastrointestinal track are reviewed. In **figure 1** an overview of the factors affecting the composition of the gut microbiota throughout life is presented.

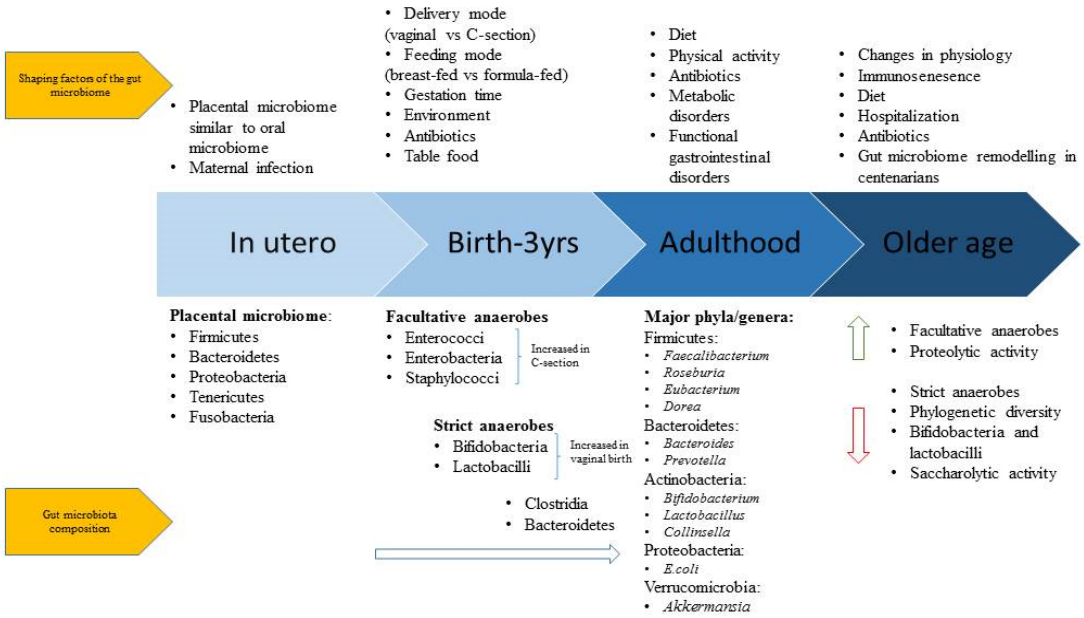


Figure 1 Overview of the Overview of the factors affecting the composition of the gut microbiota throughout life. C-section: Caesarean section, *E. coli*: *Escherichia coli*.

1.1.3 Establishment of the gut microbiota

Several studies on germ-free (GF) mice demonstrate the importance of the gut microbiota in the development of gut physiology and the priming of the innate and adaptive immune systems (reviewed in Sekirov *et al*., 2010; Sommer and Backhead, 2013). Disrupted colonisation patterns of the GIT in early life may have an immediate or future impact on health (Salminen *et al*., 2004; Penders *et al*., 2007; Cahenzli *et al*., 2013; El Aidy *et al*., 2013; Penders *et al*., 2013; Ardeshir *et al*., 2014).

In utero, the foetus is generally considered sterile although some recent studies suggest the opposite. Bacteria such as *Escherichia coli*, *Enterococcus faecium* and *Streptococcus epidermidis* have been isolated from the meconium of healthy term infants (Jimenez *et al*., 2008). Bacteria have also been isolated from the amniotic

147 fluid of preterm neonates which could however be indicative of a hidden infection
148 and preterm delivery risk (DiGiulio *et al*., 2008). In a first study on the placental
149 microbiome, Aagaard *et al*. (2014) conducted 16S rRNA sequencing and shot-gun
150 metagenomics on placental samples from 320 individuals. Comparing the findings to
151 other body microbiota data they reported the presence of a distinct community
152 comprised of non-pathogenic Firmicutes, Tennericutes, Proteobacteria, Bacteroidetes
153 and Fusobacteria. The placental microbiota was found to be similar to the microbiota
154 of the oral cavity. Importantly, an association between placental microbiota and
155 infection during the first pregnancy months and risk of preterm birth was observed.
156 Despite data suggestive of prenatal presence of bacteria in the uterus environment,
157 the role of these bacteria in the colonisation of the gastrointestinal track (GIT)
158 remains largely unclear and debateable (Matamoros *et al*., 2013). Recently, Perez-
159 Muñoz (2017) concluded that the existing data connot support the pre-birth existence
160 of fetal microbiota.

161 Colonisation of the human GIT is a dynamic and complex event and begins with
162 facultative anaerobes that prime the niche for the strict anaerobes that will follow
163 and ultimately prevail in the healthy adult microbiota. After birth the first bacteria
164 that colonise the infant gut are Enterococci, Enterobacteria (*E. coli*, *E. faecium*,
165 *Enterococcus faecalis*) and some staphylococci, and when these organisms deplete
166 the gut of oxygen anaerobes such as bifidobacteria, lactobacilli, clostridia and
167 Bacteroidetes emerge (Palmer *et al*., 2007; Alderberth *et al*., 2009). In the
168 repopulation of a GF murine model with normal murine gut microbiota, fermentation
169 metabolites and cross-feeding between early colonising bacteria and successors
170 facilitated the establishment of the latter (El Aidy *et al*., 2013). The colonisation
171 event is regarded as leading to a dynamic eubiotic balance between the commensal

bacteria and the host inhibiting the growth of pathobionts, a phenomenon called colonization resistance (Lawley and Walker, 2012).

It has been observed that in the first days of life the microbiota not only in the gut but in all body niches (skin, oral mucosa, nasopharyngeal cavity) is similar and reflects the mode of delivery (Dominguez-Bello *et al*., 2010). Samples taken from new-borns within 24 h of birth showed that vaginally born neonates harboured a microbiota close to the maternal vaginal microbiota with *Lactobacillus, Prevotella*, *Atopobium* and *Sneathia* spp. as prevalent taxa, whereas in neonates delivered by Caesarean section (C-section) the microbiota of various body niches including the gut resembled that of maternal skin (Dominguez-Bello *et al*., 2010). A few days after birth bifidobacteria are among the most dominant taxa in the gut of naturally delivered babies (Biasucci *et al*., 2008) but they seem to be absent or in low representation in the gut of new-borns delivered by C-section (Aires *et al*., 2011).

Jost *et al*. (2012) found that from the fourth day of life till the end of the first month the gut microbiota of vaginally delivered breast-fed babies was dominated by bifidobacteria. It was also reported that *Bacteroides* species showed variant patterns of establishment that correlated to low counts of *Bifidobacterium* species, that clostridia of the clusters IV and XIV were undetectable and that the presence of *Lactobacillus* was not stable (Jost *et al*., 2012). Other studies also confirm that *Bifidobacterium* and *Lactobacillus* dominate the infant gut (Turroni *et al*., 2012). Aspects of the colonisation of the human gut by bifidobacteria have been comprehensively reviewed in Ventura *et al*. (2012).

Interestingly, gestation time seems to affect the colonisation patterns of the GIT. Vaginally delivered preterm babies seem to have an intestinal microbiota of low

196 phylogenetic diversity compared to full-term infants (Arboleya *et al*., 2012). In
197 comparison to full-term babies preterm children were found to harbour more
198 facultative and fewer strict anaerobes and these differences could be attributed to
199 exposure to intensive medical treatment, hospitalisation and insufficient organ
200 function (Arboleya *et al*., 2012).

201 The gut microbiota of preterm infants seems to be a risk factor for the development
202 of necrotising enterocolitis (NEC) / late onset sepsis (LOS). Both culture and non-
203 culture based analysis of stool samples from infants born around the 27th week of
204 pregnancy showed that those diagnosed with NEC and LOS had higher faecal
205 abundance of Enterobacteria and coagulase negative staphylococci (CONS)
206 respectively (Stewart *et al*., 2012). As observed in another study on the gut
207 microbiota of preterm babies (Mshvildadze *et al*., 2010) meconium was not sterile.
208 Dysbiosis rather than the presence of pathobionts, for example CONS, was
209 postulated to be significant in the onset of LOS in preterm patients (Mai *et al*., 2013).
210 The group also observed low abundance of bifidobacteria in stool samples of LOS
211 patients. Interestingly, it has been recently suggested that irrespective of mode of
212 birth, feeding and antibiotic treatment the colonisation of the preterm GIT follows a
213 certain pattern from Bacilli to γ-Proteobacteria to Clostridia mainly influenced by
214 time of birth (La Rosa *et al*., 2014).

215 It has been controversially proposed that during the final pregnancy stages maternal
216 symbiotic gut bacteria could translocate to the mammary gland (Fernandez *et al*.,
217 2013). Studies identified *Streptococcus*, *Staphylococcus* and *Corynebacterium*, and
218 lactic acid bacteria and bifidobacteria as dominant bacteria in the breast-milk
219 microbiota which however, seems to change with lactation towards a community

220 composition more rich in *Veillonella*, *Leptotrichia* and *Prevotella* (Hunt *et al*., 2010;
221 Cabrera-Rubio *et al*., 2012; Soto *et al*., 2014). The same strains of bifidobacteria,
222 lactobacilli and staphylococci have been isolated from both the faeces of new-borns
223 and the maternal breast-milk of their mothers (Martin *et al*., 2012) demonstrating
224 that the two bacterial communities are interrelated.

225 Many studies have confirmed that the intestinal microbiota of breast-fed new-born
226 children harbours more bifidobacteria than formula-fed e.g. Harmsen *et al*. (2000),
227 Martin *et al*. (2008), Roger *et al*. (2010), Bezirtzoglou *et al*. (2011). Others failed to
228 report significant differences in the bifidobacteria population in infant stool samples
229 between breastfed and formula-fed babies but abundance of *E. coli* and *Clostridium*
230 *difficile* was higher in formula-fed subjects (Penders *et al*., 2005).

231 Breast milk contains human milk oligosaccharides (HMO). New-born babies are not
232 able to fully digest those oligosaccharides but infant-type bifidobacteria like
233 *Bifidobacterium longum* subsp. *infantis* are genetically endowed with the ability to
234 efficiently metabolise various short chain HMOs, an ability not detected in adult-
235 type bifidobacteria like *B*. *longum* subsp. *longum* (LoCassio *et al*., 2010; Sela and
236 Mills, 2010; Garrido *et al*., 2011). A variety of pathways for HMO metabolism are
237 distributed among the infant-type bifidobacteria revealing the selective pressure
238 breast feeding has put on certain bacteria groups (Zivkovic *et al*., 2011). In all cases,
239 research agrees that after weaning, the introduction of solid food marks clear
240 changes in the microbiota although pre-weaning colonisation patterns are still
241 detectable (Fallani *et al*., 2011; Koening *et al*., 2011).

242 As discussed below, antibiotics have an impact on the gut microbiota at every age.
243 Analysis of faecal microbiota of infants exposed to antibiotic treatment during the

first days after birth showed that the microbiota had limited numbers of bifidobacteria and high abundance of Enterobacteria and Proteobacteria (Brunser *et al*., 2006; Tanaka *et al*., 2009; Persaud *et al*., 2014). In early life antibiotics can disturb normal colonisation patterns which in turn can affect mammalian immunity and metabolism in later life (reviewed in Rautava *et al*., 2012). Cho *et al*. (2012) observed that infant mice treated with antibiotics developed a gut microbiota that was correlated to increased body fat. Cox *et al*. (2014) showed that in weaning mice antibiotic treatment resulted in an altered gut microbiota capable of inducing obesity, a permanent trait of the microbiota that could be transmitted through faecal transfer to GF mice. The group postulated that the microbiota-related obesity was a result of the altered gut microbiota composition due to antibiotic treatment. This has profound implications for human weight management at population levels because paediatric antibiotic usage is very frequent.

Studies suggest that the microbiota begins to stabilise to a more adult-like profile from year one till 2.5 years of age (Palmer *et al*., 2007; Koening *et al*., 2011). More recent research shows that the microbiota undergoes changes till the 36th month of age and that an enterotype establishment can be observed between the 9th month and the third year of age (Yatsunenko *et al*., 2012; Bergstrom *et al*., 2014).

1.1.4 Shaping factors of gut microbiota composition

1.1.4.1 Diet

Habitual diet plays a significant role in the development of the composition of the intestinal microbiota (Scott *et al*., 2013, Simoes *et al*., 2013). The intestinal microbiota rapidly responds to diet variations which is a characteristic of the gut microbiota of many mammalian groups (Muegge *et al*., 2011; David *et al*., 2014).

Most of the dietary nutrient absorption occurs in the duodenum and small intestine whereas undigested molecules like complex polysaccharides reach the large intestine (reviewed in Wong and Jenkins, 2007). In the colon a phylogenetically diverse and metabolically active bacterial community salvages energy from the undigested food particles which is subsequently used for the maintenance of the microbiota and by the host (Blaut and Clavel, 2007).

Fermentation of undigested polysaccharides as well as dietary and endogenous proteinaceous substrates and polyphenols results in various end-products with tremendous impact on colonic health and homeostasis (Cummings and Macfarlane, 1997; Nyangale *et al*., 2012, Windey *et al*., 2012). These fermentation compounds are predominantly the short chain fatty acids (SCFA) propionate, acetate and butyrate, and gasses like H_2, H_2S and CO_2 as well as potentially toxic phenolic and indolic compounds, ammonia, amines and sulphur molecules (reviewed in Macfarlane and Macfarlane, 2012).

Different non-digestible carbohydrates like resistant starch (RS), non-starch polysaccharides (NSP) and prebiotics can selectively enhance the growth of certain bacterial groups in the GIT (Martinez *et al*., 2010; Walker *et al*., 2011). Modulated microbial fermentation can yield beneficial end-products for the host. One such fermentation product is butyrate that is extensively studied for the beneficial effect it exerts on colonic epithelial cells (reviewed in Hamer *et al*. 2008). *Clostridium* clusters XIVa and IV, with representatives like *Eubacterium* / *Roseburia* species and *F. prausnitzii* respectively, are major saccharolytic players in butyrate generation in the colon (reviewed in Louis and Flint, 2009) and potentially prominent regulators of

innate immunity through the production of SCFAs (Atarashi *et al*., 2011; Atarashi *et al*., 2013; Smith *et al*., 2013).

A statistical model was generated by Faith *et al*. (2011) that allowed for up to 60% accuracy in the prediction of shifts in species abundance as a response to host diet. The team isolated ten bacteria species from the human colon, sequenced their genomes and introduced them in gnotobiotic mice. They subsequently conducted detailed measurements on species abundance and gene expression in response to host diet, from which the statistical model was developed. Animal models are valuable tools in studying shifts in the microbiome but there is need for more extensive human studies to better simulate the complex conditions occurring naturally in the GIT (Walker *et al*., 2010; Muegge *et al*., 2011). Importantly, a recent study by Korpela *et al*. (2014) developed models to predict the gut microbiota responses to diet intervention based on the intestinal microbiota composition, predominantly on the Firmicutes abundance, of certain obese individuals.

A summary of culture-independent studies correlating habitual diet to gut microbiota is presented in **Table 1**. From metagenomic studies on human faecal data sets from different countries Arumugam *et al*. (2011) observed that collectively, the grouping of the data formed three clusters called "enterotypes". Analytically, enterotype number 1 was *Bacteroides*-dominated and positively co-related with *Parabacteroides*, *Lactobacillus*, Clostridiales and *Alkaliphilus* species, and was characterised by enrichment in metabolism of a broad spectrum of carbohydrate utilization and proteolytic activity. In enterotype 2 *Prevotella* was predominant and co-occurred with *Desulfovibrio* probably in a synergistic consortium for mucin degradation. The *Ruminococcus* enterotype correlated with *Akkermansia* abundance

315 which is a mucin-degrading bacterium. The "enterotype" classification was based on
316 the high abundances of certain genera and this grouping should be regarded as
317 metabolic variations in the way gut microbiota utilises available nutrients.

318 Consumption of a vegetarian diet leads to changes in the microbiota of the GIT. Low
319 abundance of *Clostridium* cluster IV and higher but not significant abundance of
320 *Bacteroides* was observed by qPCR analysis of faecal samples of vegetarians
321 compared to omnivores (Liszt *et al*., 2008). Another culture-independent study
322 reported low abundance of *Clostridium* cluster XIVa and the Roseburia / E. rectale
323 group in the faecal samples of vegetarians compared to omnivores (Kabberdoss *et al*.,
324 2012). Diet intervention with strict vegetarian diet on obese individuals for one
325 month led to changes in the intestinal microbiota composition of patients without
326 any impact on enterotypes and improved inflammatory markers in blood (Kim *et al*.,
327 2013). In that study, the diet intervention reduced the Firmicutes / Bacteroides ratio
328 and the proportion of Enterobacteria in the intestinal microbiota, and increased the
329 abundances of *Clostridium* cluster XIVa and IV.

330 Based on diet interventions and habitual diet of 98 individuals Wu *et al*. (2011)
331 reported that colonic communities clustered according to the aforementioned
332 enterotypes 1 and 2 and that number 3 demonstrated a tendency to fuse with
333 enterotype 1. The *Bacteroides*-dominated cluster co-occurred with *Alistipes* and
334 *Parabacteroides* and associated with an animal protein and saturated fat-based
335 habitual diet. In contrast, the *Prevotella*-cluster correlated with *Paraprevotella* and
336 *Catenibacterium* and was related to fibre intake. Importantly, enterotypes as
337 monitored by Wu *et al*. (2011) were driven by habitual diet and overall, remained
338 stable during the diet intervention, although changes in the microbiome did occur as

a response to the new diet. The group also concluded that the two clusters mirrored a westernised and a more agrarian kind of diet respectively.

Similar conclusions were drawn when high-through put sequencing was conducted on 16S rDNA from faecal samples of children of two geographically distinct areas: urban in Florence, Italy and rural in Boulpon, Burkina Faso (De Filippo *et al*., 2010). The rural diet was almost vegetarian and enriched in starch, fibre and plant polysaccharides and low in animal fat and protein. The faecal microbiota of subjects residing in rural areas was enriched in bacteria enabling maximum energy harvest from fibre and high rates of SCFAs production. Genera like *Xylanibacte*r, *Prevotella*, *Butyrivibrio* and *Treponema* were characteristic of the intestinal microbiota of people living in rural areas and indicative of the potential to harvest energy from complex polysaccharides. By contrast, the urban diet sustained a much less diverse microbiota.

Taken together, the results of the two studies above reveal the impact on gut microbiota of two kinds of diet, one "rural" enriched in fibre and low in fat and the other more "westernised", rich in animal protein and saturated fat. The global convergence of dietary habits towards a westernised high fat / low fibre diet is an increasing phenomenon in modern society and is accompanied by the escalating prevalence of inflammatory and metabolic disease. The interplay between Westernised dietary habits, commensal bacteria and inflammation are comprehensively reviewed by Thorburn *et al*. (2014).

1.1.4.2 Exercise

No extensive studies have been dedicated so far to the exploration of the effect of exercise on the human microbiota. There are however some indications that exercise

affects the gut environment based on a murine model. Matsumoto *et al*. (2008) observed that rats that regularly exercise on the wheel-run had an intestinal microbiota distinct from the microbiota of sedentary animals and notably the former had higher colonic butyrate concentration compared to sedentary littermates. Choi *et al*. (2013) observed exercise-induced changes in the intestinal microbiota of healthy mice. Another study showed that exercise can lead to shifts in the gut microbiota of obese mice (Evans *et al* 2013). Recently, Clarke *et al*. (2014) studied the effect of exercise on the intestinal microbiota of the members of a professional rugby team. The study showed that athletes had a distinct faecal microbiota and lower inflammatory status in blood compared to controls. Athletes and low body mass index (BMI) controls had higher abundance of *Akkermansia* in the faecal samples compared to high BMI controls. The group correlated the gut microbiota changes not only with regular intense exercise but with the high protein diet regime the athletes consumed. However, it was impossible to rule out the effect of potential confounding factors. The existing evidence indicate that gut-microbiota is responsive to exercise but more research including intervention studies is necessary to elucidate the mechanisms of such responses. Recently, Barton *et al.* (2018) reported that after reexaminig the microbiome of the Clarke *et al*. (2014) study-participants using shotgun metagenomics analysis, the microbiome of athletes at taxonomic and mostly at metabolomic level had significant differences compared to the more sendetary controls; the athletes' microbiome profile was associated with high fibre and protein diet and exercise.

385 **Table 1 Culture-independent studies associating changes in gut microbiota to diet.**

Cohort	Analysis Method	Diet	Main findings	Reference
29 healthy adults	qPCR, PCR-DGGE	Habitual vegetarians, omnivores	↓*Clostridium* cluster IV ↑*Bacteroides* spp. (non-significant)	Liszt *et al*. (2009)
46 healthy adults	qPCR	Habitual vegetarians, omnivores	↓Clostridium cluster XIVa ↓*Roseburia* / *E. rectale* group	Kabberdoss *et al*. (2012)
6 obese with type 2 diabetes (T2D)	16S rRNA sequencing, biochemical analysis	Strict vegetarian diet/ 1 month	↓Firmicutes/Bacteroides ratio ↓No effect on enterotypes ↓Pathobionts, Enterobacteria ↑Bacteroides fragilis, Clostridium cluster XIVa, IV species ↓Body weight, blood triglycerides, cholesterol	Kim *et al*. (2013)
14 healthy Mossi ethnic group children (rural community), 15 healthy Europeans (urban community)	16S rRNA sequencing	-Habitual rural diet: low fat, protein, sugar, high fibre -Habitual urban diet: high fat, protein, sugar, low fibre	-↑Bacteroidetes, unique presence of *Xylanibacter*, *Prevotella*, *Butyrivibrio*, *Treponema* -↑Firmicutes, ↑Enterobacteria	De Filippo *et al*. (2010)

Cohort	Analysis Method	Diet	Main findings	Reference
-98 healthy subjects	16S rRNA sequencing	-Habitual fat-rich diet -Habitual fibre-rich diet	-↑*Bacteroides*, *Alistipes*, *Parabacteroides* -↑*Prevotella*, *Paraprevotella*, *Catenibacterium*	Wu *et al*. (2011)
-10 healthy *Bacteroides* enterotype		Diet intervention: -high fat/protein -carbohydrates	-*Bacteroides* enterotype correlated -*Prevotella* enterotype related -No permanent enterotype switch	
14 overweight subjects	16S rRNA sequencing, qPCR	-Resistant starch diet intervention	-↑*Roseburia* / *E. rectale* related Firmicutes	Walker *et al*. (2011)
		-Reduced carbohydrates diet intervention	-↓*Roseburia* / *E. rectale* related Firmicutes	
18 lean subjects, 33 mammalian species	16S rRNA sequencing, qPCR	Proteins, insoluble fibre	-Similar enzymatic activity and adaptation to diet across species	Muegge *et al*. (2011)
178 older subject: community, long-stay	16S rRNA sequencing	-Community: moderate fat/high fibre, diverse diet	-↑Phylogenetic diversity, ↑Firmicutes, *Roseburia*, *Coprococcuss*	Claesson *et al*. (2012)
		-Long-stay: high fat/protein/sugar, low fibre, less diverse diet	-↓Phylogenetic diversity, ↑Bacterodetes, *Parabacteroide,* ↓*Eubacterium*, A*naerotruncus*, *Coprobacillus*	
20 monozygotic twins	16S rRNA sequencing, DGGE	Habitual diet	*Bacteroides* spp. and bifidobacteria most affected by habitual diet	Simoes *et al*. (2013)

Cohort	Analysis Method	Diet	Main findings	Reference
40 professional athletes	16S rRNA sequencing, biochemical	Strict habitual diet, high protein	↑*Akkermansia* sp., ↓inflammatory markers in blood	Clarke *et al*. (2014)
10 healthy individuals	16S rRNA sequencing, qPCR, RNA-sequencing, biochemical	Short-term intervention: -Animal-based diet -Plan-based diet:	 -↑Bile-tolerant bacteria: *Alistipes*, *Bilophila*, *Bacteroides,* ↓plant fibre metabolising Firmicutes -↑*Roseburia / Eubacterium,* Ruminococci	David *et al*. (2014)

1.1.4.3 Antibiotics

Antibiotic treatment can reduce the compositional diversity and the metabolic potential of the gut microbiome, with an effect that attenuates with treatment cessation (Pérez-Cobas *et al*., 2013). Jakobsson *et al*. (2010) studied subjects receiving clarithromycin and metronidazole treatment and monitored microbiota over a period of four years. After a week of treatment distinct changes in the composition of the intestinal microbiota of all subjects were observed. Interestingly, the group also observed that although the general trend was gut microbiota recovery in individuals after completion of treatment, some cases failed to fully recover the pre-treatment microbiota. The same pattern of inter-individual responses to antibiotics, recovery after cessation of treatment but with cases among subjects studied with reduced to absent restoration of taxa in the intestinal microbiota, was reported in other studies (Jernberg *et al*., 2007; Dethlefsen *et al*., 2008; Dethlefsen and Relman, 2011). Inter-individual responses reflected the variations of the healthy status microbiota among individuals. The faecal metabolome associated with the microbiota seems to be affected. Streptomycin was shown to exert multiple effects on murine intestinal metabolic activity (Caetano *et al*., 2011) implying a potential effect on the microbiota involved in certain metabolic pathways

Low diversity of the gut microbiota makes the intestinal environment susceptible to entro-pathogens. In a murine model after two days of treatment with vancomycin/streptomycin a dose-dependent effect was apparent upon the composition of the gut microbiota (Sekirov *et al*., 2008). The group observed that although total bacterial numbers were not affected, the composition of the microbiota was altered making the animals vulnerable to *Salmonella* serovar *Typhimurium* infection.

Another common example of infection susceptibility and breach in the colonisation resistance phenomenon is that of *Clostridium difficile* infection (CDI). *C. difficile* is a common nosocomial pathogen, causing antibiotic-associated diarrhoea (AAD) and affecting mostly hospitalised older patients. Epidemiology and risk factors of CDI were reviewed by Ananthakrishnan (2011). Cephalosporins, macrolides, clindamycin and predominantly fluoroquinolones are some of the associated infection-risk antibiotics (Pépin *et al*., 2005). The successful treatment of CDI in a mouse model with a mixture of bacteria isolated from healthy subjects (Lawley *et al*., 2012), and in humans with a stool substitute (Petrof *et al*., 2013) showed that a phylogenetically diverse mix of faecal bacteria is sufficient to displace *C. difficile* from the gut more effectively than any known antibiotic.

Overall, antibiotic administration results in mostly short-term disturbances in the microbiota and these disturbances tend to revert with the end of antibiotic use. The dominant taxa in the gut microbiota tend to recover soon after short-term administration ends showing that balancing ecological mechanisms drive the microbiota back to its pre-treatment status whereas long-term use may result in permanent alterations in the intestinal microbiota (De La Cochetiere *et al*., 2005) and increase risk of AAD.

1.1.4.4 Extra-intestinal disorders: Metabolic syndrome and related obesity, type 2 diabetes, liver disease

The metabolic syndrome is mainly characterised by insulin resistance and visceral obesity as well as raised blood pressure, atherogenic dyslipidaemia and pro-inflammatory status (Grundy *et al*., 2004). The syndrome is a risk factor for the interrelated development of cardiovascular disease (CDV) and type 2 diabetes (T2D) (Huang, 2009). Dysbiosis seems to play a role in this syndrome. The intermediate

436 step is the interplay between the gut microbiota, nutrient absorption, fat storage and
437 host metabolism (reviewed in Musso *et al*., 2011). The metabolic syndrome also
438 affects hepatic physiology with the potential for development of the non-alcoholic
439 fatty liver disease (NAFLD) and the more severe non-alcoholic steatohepatitis
440 (NASH) (Abu-Shanab and Quigley, 2010).

441 **Obesity and type 2 diabetes**

442 There is an accumulation of evidence that commensal bacteria play a significant role
443 in modulating fat storage. When lean GF mice were conventionalised with normal
444 gut microbiota they gained body fat although food intake was not increased
445 (Backhed *et al*., 2004). Cani *et al*. (2007; 2008) used a murine model to propose a
446 sequence of events leading from high-fat diet to gut microbiota-controlled metabolic
447 disorders. High-fat feeding was correlated with increased intestinal permeability and
448 high lipopolysaccharides (LPS) plasma concentrations leading to metabolic
449 endotoxemia, inflammation and eventually metabolic conditions. Obesity and
450 diabetes reflecting the metabolic deregulation were attenuated in a mouse strain
451 lacking the main LPS-receptor CD14, demonstrating the importance of LPS in the
452 initiation of the metabolic deregulation (Cani *et al*., 2007). In humans, plasma LPS
453 levels increase after increase of fat intake (Erridge *et al*., 2007) and translocation of
454 bacterial components across the gut barrier seem to occur in obese and diabetic
455 subjects (Gummesson, *et al*., 2011; Hawkesworth *et al*., 2013).

456 Importantly, LPS interacts with the endocannabinoid system (eCB) which emerges
457 as an important player in the metabolic deregulation in obesity (Lamber and
458 Muccioli, 2007). The endocannabinoid system is implicated in gut barrier integrity
459 as blocking the G protein-coupled receptor of eCB CB_1 resulted in reduced plasma

460 LPS and an increase in tight-junction proteins in murine model (Muccioli *et al*.
461 2010).

462 Ley *et al*. (2005) showed that the establishment of the diet-induced obesity
463 phenotype in mice was accompanied by low intestinal microbiota diversity and
464 altered relative abundances of the major taxa with increased abundance of Firmicutes
465 and reduction to 50% of phylum Bacteroidetes compared to lean mice. The group
466 also demonstrated that the potential to gain energy from nutrients was improved in
467 this "obese microbiome": more enzymes involved in starch and carbohydrate
468 metabolism, more fermentation-end products like butyrate and reduced energy load
469 in faeces compared to lean littermates (Turnbaugh *et al*., 2006). Importantly, the
470 increased energy uptake trait could be transmitted to germ-free mice by faecal
471 transplantation (Turnbaugh *et al*., 2006). Studying obesity-associated shifts in gut
472 microbiota in humans, Ley *et al* (2006) obtained results analogous to data from the
473 mouse studies. Accordingly, in obese humans Bacteroidetes relative abundance was
474 lower compared to Firmicutes. However, studies by other groups in humans have
475 been contradictory and report different changes in the ratios of the Firmicutes and
476 Bacteroidetes (Duncan *et al*., 2008; Schwiertz *et al*., 2010). In a Danish cohort of
477 obese and non-obese people, metagenomics analysis showed that the number of the
478 gut microbiota genes used as a measure of bacterial richness in the microbiota, was
479 associated with obesity phenotypes (Le Chatelier *et al*. 2013). In individuals with
480 low gene counts demonstrated associated obesity phenotypes such adiposity and
481 insulin resistance, and had increased abundance of *Bacteroides*, *Parabacteroides,*
482 certain *Ruminocccus* taxa and Proteobacteria among others, whereas in the gut
483 microbiota of individuals with high gene counts "anti-inflammatory" taxa such as
484 *Faecalibacterium* were dominant (Le Chatelier et *al*. 2013). Cotillard *et al.* (2013)

reported that low gene richness in obese and overweight individuals was associated with higher low-grade inflammation and deregulated metabolism.

Like in obesity, research on the microbiota of patients with type 2 diabetes (T2D) reveals significant alterations compared to healthy controls. The "diabetic microbiota" had high proportions of Bacteroidetes-*Prevotella* group whereas the abundance of the butyrate-producer group *C. coccoides-E. rectale* was reduced (Larsen *et al*., 2010). A metagenomic analysis on the faecal microbiome of Chinese T2D patients showed decreased butyrate biosynthesis and metabolism of vitamins, enrichment in metabolism of xenobiotics, branched chain amino acids (BCAA) and methane, and sulphate reduction to mention some (Qin *et al*., 2012). The group also reported opportunistic pathogens, the mucin-degrader *Akkermansia municiphila* and sulphur reducers like *Desulfovibrio* sp. to be higher in abundance in T2D faecal microbiota. A metagenome-wide study on a cohort of 145 healthy and with risk for diabetes European women also revealed composition and function alterations in the gut microbiota of the at-risk subjects (Karlsson *et al*., 2013). The group developed a diabetes risk predicting tool which however failed to predict diabetes when applied on the aforementioned Chinese cohort suggesting that the metagenomic markers for T2D between the two cohorts were cohort-specific and could not be generalised. Importantly, Forslund *et al*. (2015) reported that when controlling for the use of antidiabetes drug use i.e. metformin, a certain "diabetic" gut microbiota profile emerged characterised by significant reduction in butyrate producers. Metformin is widely used for T2D treatment and there is data supporting that part of its antidiabetic activity is mediated by acting on the gut micorbiota (Wu *et al*., 2017).

Non-alcoholic fatty liver disease (NAFLD), non-alcoholic steatohepatitis (NASH)

A range of hepatic conditions related to obesity are grouped under the term NAFLD including the inflammatory steatosis NASH (Brunt and Tiniakos, 2010). In obesity accompanied by co-morbidity the NAFLD prevalence is 95% and prevalence for NASH is 25%, whereas in diabetic patients NASH may reach 63% prevalence (Bajaj *et al*., 2012). The gut microbiota of patients with either NAFLD or NASH have not yet been extensively studied.

Mouzaki *et al* (2013) conducted one of the first culture-independent studies on 50 adults with steatosis, non-alcoholic steatohepatitis and healthy controls. The group observed a diet-independent reduced relative abundance of Bacteroidetes in the stool of NASH patients. A previous study on the intestinal microbiota of obese, healthy children and children with NASH showed that obese and NASH microbiomes could cluster in a *Prevotella*-enriched enterotype diversifying from the healthy controls (Zhu *et al*., 2012). *Bacteroides* abundance was higher in obese and NASH subjects compared to healthy controls, Actinobacteria had relatively lower abundance in non-healthy subjects and Proteobacteria abundance increased significantly from healthy to obese individuals with higher scores in NASH subjects (Zhu *et al*., 2012). The study also associated the elevated ethanol measurements in NASH patients blood samples to a gut microbiota enriched in ethanol-producing bacteria like *Escherichia coli*.

Data on the gut microbiota of NAFLD patients is still limited. A clear link between gut microbiota, diet and metabolic disorders exist. However, these two studies on NAFLD and intestinal microbiota yielded diet-independent results implying a causal role of the gut microbiota in the pathogenesis of the condition. Serino *et al*. (2012) investigating metabolic disorders and gut microbiota in a murine model showed that

irrespective of diet and host genetics gut microbiota and the naturally occurring metabolic variations of the intestinal microbiome among individuals predisposed the animals for metabolic conditions. Future work is expected to further elucidate both the mechanisms through which diet controls gut microbiota-driven host metabolism disorders and the existence of a diet-independent role of the gut microbiota in the progression of metabolic conditions.

1.1.4.5 Functional gastrointestinal disorders: Inflammatory bowel disease (IBD), Irritable bowel syndrome (IBS)

Functional gastrointestinal disorders (FGIDs) are a broad group of commonly occurring gastrointestinal conditions with irritable bowel syndrome (IBS) being the most prevalent (Talley, 2008). Using Rome III criteria similar symptoms in IBS and IBD could be identified (Bryant *et al*., 2011).

Inflammatory bowel disease (IBD)

The two major forms of IBD are ulcerative colitis (UC) and Crohn's disease (CD) and both are influenced by host genetics and deregulated immunological response to the intestinal microbiota (Abraham and Cho, 2009). Culture-independent studies show abnormalities in the microbiome of individuals with IBD. Frank *et al*. (2007) compared the microbiota in biopsies of UC and CD to those of healthy controls. Although the abundances of the predominant taxa were not altered, significant variations in the relative abundances of subgroups distinguished the healthy from the non-healthy microbiotas and health-promoting butyrate-producers belonging to Bacteroidetes and Lachnospiraceae were significantly reduced in abundance in the IBD samples. Conversely, Proteobacteria and *Bacillus* species were increased in proportion.

Manichah *et al* (2006) applied metagenomics to the faecal microbiota of CD patients and observed reduced phylogenetic diversity. Patient faecal samples demonstrated low abundance of the butyrate-producer groups *Clostridium leptum* and *Clostridium coccoides* and were enriched in Gram negative species and the family *Porphyromonadaceae. F. prausnitzii* is a major butyrate-producer and the dysbiosis and symptomology characterized by the significant reduction of this Firmicutes in CD could be reversed by oral administration of this organism in a murine model (Sokol *et al*., 2008).

Another bacterium implicated in IBD is *Bacteroides fragilis*. Transcriptional analysis of colonic mucosal biopsies showed that the phylum Bacteroidetes was the most active based on rRNA gene libraries and *B. fragilis* was dominant in CD based on proportion of sequences in rRNA library/percentage of sequences in rRNA gene library (AIR) (Rehman *et al*. 2010). Previously, Swidsinski *et al*. (2005) had demonstrated the prevalence of this species in gut microbiota of IBD patients and especially CD and they suggested the potential role of the high abundance of this bacterium in increased barrier permeability.

Risk factors for IBD have been identified, and host genetics and dysbiosis have been associated to the progression of the condition. However, the exact mechanisms leading to IBD remain elusive thus making the unravelling of IBD causation a paradigm for the understanding of the complex interplay between microbiota, host and inflammation (Huttenhower *et al*., 2014). Furthermore, the complex cross-talk between the causative factors of the condition, the inter-individual variations in gut microbiota and the fact that no universal colitogenic bacteria have been identified

580 indicate the need for more personalised IBD therapeutic strategies (Stephens and
581 Round, 2014).

582 **Irritable bowel syndrome (IBS)**

583 A common FGID disorder is IBS and unlike IBD it is not associated with
584 histopathological conditions and it is believed to be related to the deregulation of the
585 gut-brain axis (GBA) (Ringel and Maharshak, 2013). The cross-talk between gut and
586 brain and the association with intestinal homeostasis and progression of FGID have
587 been recently comprehensively reviewed by Collins and Bercik (2009) and Collins
588 (2014). IBS progression can be outlined as follows: extrinsically-induced dysbiosis,
589 for example after severe infection, triggers mucosal immunity in genetically
590 predisposed subjects resulting in enteric nervous system deregulation caused by
591 intestinal permeability (Collins *et al*., 2009; Simren *et al.* 2009; Steck *et al*., 2013).

592 The diagnostic criteria set by the Rome Foundation, Rome criteria III
593 (http://www.theromefoundation.org/criteria/), are widely used for FGID diagnosis
594 (Soares, 2014); outlined here in **Table 2**. Three subtypes of the condition exist based
595 on stool appearance: diarrhoea-predominant (IBS-D), constipation-predominant
596 (IBS-C), mixed (IBS-M) (Longstreth *et al*., 2006).

597 Using culture-based techniques and PCR - denaturing gradient gel electrophoresis
598 (PCR-DGGE) analysis Matto *et al*. (2005) observed temporal instability of the gut
599 microbiota of IBS patients but failed to associate shifts in bacterial groups with
600 symptoms of the condition. Other culture-independent studies also showed temporal
601 instability of the intestinal microbiota of IBS subjects, lower abundance of
602 *Lactobacillus* species in stool samples of patients with IBS-D, increase in abundance

603 of *Veillonella* species in IBS-C individuals and a reduction in Clostridia species
604 abundance (Malinen *et al*., 2005; Kassinen *et al*., 2007).

605 Other culture-independent studies showed that in the faecal microbiota of IBS
606 patients compared to controls, the Firmicutes to Bacteroidetes ratio was increased,
607 Actinobacteria had lower abundance and Proteobacteria were more abundant
608 (Krogius-Kurikka *et al*., 2009; Rajilic-Stojanovic *et al*., 2011). Similar results were
609 published by Jeffrey *et al*. (2012), that is, significant differences between gut
610 microbiota of IBS patients and healthy controls and increased Firmicutes to
611 Bacteroidetes ratio. Subgroups were formed based on the faecal microbiota of
612 patients and importantly, one of these was characterised by normal-resembling faecal
613 microbiota. These patients had high scores for anxiety and depression, suggesting a
614 subclass of IBS on non-microbial aetiology.

615 In stool samples of IBS-C patients the fermentation potential of the microbiota was
616 altered: fluorescent *in situ* hybridisation (FISH) revealed that the abundance of H_2-
617 consuming sulphur-reducing bacteria and butyrate producing *Roseburia* / *E. rectale*
618 group was significantly higher and lower respectively compared to control subjects
619 (Chassard *et al*. 2012). The altered fermentation activity of the intestinal microbiota
620 in IBS patients was also indicated by abnormal hydrogen and methane breath
621 measurements in patients (King *et al*., 1998). Interestingly, the commensal
622 methanogenic Achaea with the production of H_2 may have a role in IBS
623 pathogenesis (Triantafyllou *et al*., 2014) but studies to date yield contradictory
624 results (Rajilic-Stojanovic *et al*., 2011; Kim *et al*. 2012).

625 Studies so far have not fully revealed the exact mechanism through which the
626 microbiota influences the onset and progression of IBS. The fact that probiotics seem

to ameliorate the symptoms of the dysbiotic condition of this multifaceted syndrome further promotes the idea of microbiota disturbances implicated in the disease (Santos and Whorwell, 2014). IBS is an interesting field of research as the unravelling of the disease mechanisms will enable further insight in the brain-gut axis communication and how mediators from the intestinal microbiota can influence cognitive function in subjects with intestinal disorders (Bercik *et al*., 2011).

Table 2 Diagnostic criteria for IBS adapted from Rome III (http://www.theromefoundation.org/criteria/).

Criteria for diagnosis of IBS	
• Intermittent abdominal pain and discomfort related to at least two of the conditions below: 1. *Improvement with defecation* 2. *Alterations in the defecation habits* 3. *Alterations in stool form*	• Frequency of symptoms: 3 days per month for the last 3 months • Symptom detection at least 6 months before diagnosis.

1.1.5 Ageing and microbiota alterations

There is growing scientific interest in the connection between low-grade inflammation observed in older age and the role of gut microbiota. According to this concept, accumulation of antigenic stimulation and stressors throughout life leads to declined immune responses and low immune adaptiveness resulting in inflamm-ageing (Franceschi *et al*., 2000). An overview of how the protective activity of the immune system (IS) is deregulated with immuno-senescence, that is the ageing of the IS, is presented by De Martinis *et al*. (2005). The Nuclear Factor κB (NF-κB) seems to be at the core of this process by being the major signalling pathway in the innate IS (Salminen *et al*., 2008).

It is now accepted that this low-grade inflammatory status with characteristic markers circulating in the plasma of older people is associated with the degenerative conditions that characterise old age (Howcroft *et al*., 2013). Among the most usual pro-inflammatory markers correlated with muscle tissue damage and sarcopenia, neuro-degeneration and obesity are C-reactive protein (CRP), tumour necrosis factor-α (TNF-α), IL-6 and IL-18 (Howcroft *et al*., 2013).

Antigenic stimuli associated with the microbiota composition shifts observed in older age may contribute to inflamm-ageing. The exact correlation and mechanisms that link the microbiome to ageing remain unclear and this presents a promising field for future research and new therapeutic strategies.

Changes in the gut microbiota in older age

Changes in the GIT physiology, in the functionality of the IS, in lifestyle and nutritional habits, and hospitalisation and medical treatment can impact the composition of the gut microbiota in older age and consequently health status (reviewed in Cusack and O'Toole, 2013).

The combined data from older mainly culture-dependent studies suggest that age is accompanied by an increase in the proportion of facultative anaerobes, with a simultaneous decline of beneficial anaerobes such as bifidobacterial and lactobacilli and importantly, an overall decline in species diversity in several bacterial groups (Woodmansey, 2007). During inflammation pathobionts - potentially harmful bacteria that are in low abundance in the healthy gut, increase in abundance displacing commensals (Pédron and Sansonetti, 2008). The example of the proliferation of the pathogenic *Salmonella enterica* serotype *Typhimurium* in the gut shows how a pathogen can compete with commensals and prevail in the dysbiotic

condition of the inflamed ecosystem and further promote inflammation (Winter *et al*., 2010). Furthermore, an alarming correlation exists between the low diversity profile of the intestinal microbiota of older subjects and infection or non-symptomatic colonization by the common nosocomial pathogen *C. difficile* (Rea *et al*., 2012).

Studies based on culture-independent techniques offered more comprehensive information about the shifts occurring in the gut microbiome of older subjects. Overall, studies do not yield homogenous results and there are variations based on the cohorts and the methods employed. However, it seems that compared to controls, the intestinal microbiota of older people is characterised by differences in the abundance of specific bacterial groups and the composition and species diversity of these groups is altered (Tiihonen *et al*., 2010). Variations in the reported composition and significant inter-individual variability in the microbiota could be attributed to the fact that ageing alone is not enough to alter the generally stable microbiota (Brussow, 2013) and the alterations should be viewed and explained accompanied by data on the cohort diet, physical activity, drug and antibiotic intake, and even geographical and socio-economical profile.

When the faecal bacterial population of older community-dwellers, hospitalised and hospitalised on antibiotic treatment were compared, Bartosch *et al*. (2004) noted that the basic difference in the faecal microbiota between healthy and hospitalized subjects was the significant reduction in the *Bacteroides* / *Prevotella* group. In the same study, bifidobacteria, *Desulfovibrio* spp., *Clostridium clostridioforme* and *F. prausnitzii* also declined in abundance after hospitalisation. However, the relative abundance of the aforementioned bacteria remained stable as the total bacterial load in stool of hospitalised subjects also decreased. Antibiotic treatment had an

693 additional negative impact on the faecal microbiota. Antibiotics lowered the
694 diversity in the stool microbiota and even eliminated certain bacterial groups,
695 promoting the growth of opportunistic species and enterococci like *E. faecalis*
696 (Bartosch *et al*., 2004).

697 Mueller *et al*. (2006) studied the gut microbiota composition of 230 healthy
698 individuals from four European countries. A significant increase in Enterobacteria
699 abundance in the stool samples of the older volunteers was observed irrespective of
700 country of origin whereas for most of the other dominant bacterial groups like *F.*
701 *prausnitzii*, bifidobacteria, *Bacteroides* / *Prevotella* no generalised conclusions could
702 be made that would unify the results from the stool samples of all individuals tested.
703 However, the *Bacteroides* / *Prevotella* group was more abundant in male subjects
704 compared to female. Zwielehner *et al*. (2009) comparing the faecal microbiota of
705 long-term residential elderly to healthy adults reported an overall decline in diversity
706 of the dominant phyla and significantly low representation of *Clostridium* cluster IV
707 and bifidobacteria along with inter-individual variations in *Bacteroides*. Comparing
708 the faecal microbiota of 161 Irish subjects aged >65 years and 9 younger controls
709 identified significant differences and remarkable inter-individual variability was
710 reported for Bacteroidetes and Firmicutes abundances (Claesson *et al*., 2011)

711 When faecal microbiota of healthy adults (around 30 years), older (around 70 years)
712 and centenarian subjects was analysed the intestinal microbiota of centenarians was
713 distinct from the other subject categories (Biagi *et al*., 2010). With Firmicutes and
714 Bacteroidetes remaining the major phyla throughout life, certain re-arrangements
715 were observed in the microbiota of centenarians with extensive remodelling in the
716 population of *Clostridium* cluster XIVa (Biagi *et al*., 2010).

Data from the ELDERMET project was able to demonstrate a clear correlation between diet, gut microbiota and health status in a large cohort of older subjects. The participants were stratified in community-dwelling and short-term and long-term hospitalized. The profiling of the intestinal microbiota was accompanied by data collection on habitual diet, inflammation status, cognitive function and frailty. Overall, subjects frequenting or residing in long-stay care units had poor diet which correlated with higher frailty scores, higher inflammation indicators like IL-6, IL-8, TNFα and CRP in the serum and a distinct microbiota in comparison to community-dwellers of the same ethno-geographic region (Claesson *et al*., 2012). Bacteroidetes-related operational taxonomic units (OTUs) were more abundant in faecal samples of long-stay subjects whereas in community-residing subjects the phylum Firmicutes was at higher levels with dominant genera *Coprococcus* and *Roseburia* for the latter group of subjects and *Parabacteroides*, *Eubacterium*, *Anaerotruncus*, *Lactonifactor* and *Coprobacillus* for the former (Claesson *et al*., 2012).

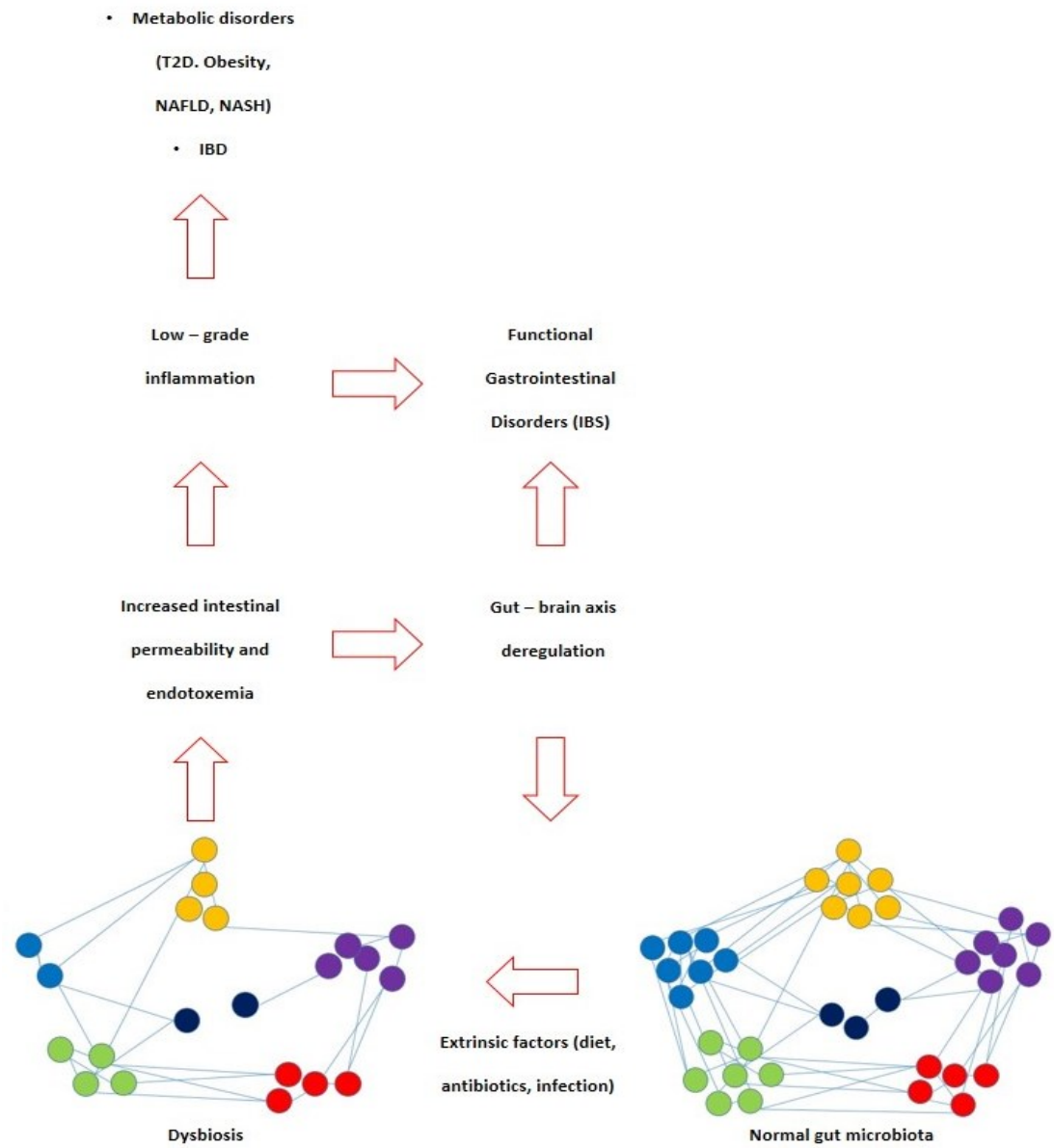
The gut microbiota in older age is characterised by both compositional and functional alterations expressed in centenarians in the marked shift from a saccharolytic metabolism towards putrefaction with significant loss of genes involved in SCFA production (Rampelli *et al*., 2013). Given the importance of these metabolites in gut homeostasis (Smith *et al*., 2013) this shift could increase the risk for disease. Protein fermentation metabolites can also be detrimental for health by affecting gut homeostasis (Windey *et al*., 2012). However, in people reaching the hundredth decade of life changes in the microbiome could be seen as an evolutionary advantage. In centenarians high phenylacetyglutamine (PAG) and p-cresol sulphate (PCS) secretion indicates shifts in the microbiome which could reflect an overall

741 remodelling of structure and functionality in order for the human organism to survive
742 in the extremities of life-spam (Collino *et al*., 2013).

743 ### 1.1.6 Concluding remarks

744 As summarised here there is accumulating evidence predominantly from studies
745 based on high throughput parallel sequencing which underlines the significance of
746 gut microbiota in modulating health and disease. The gut microbiota is affected by
747 the host life-style, with a major factor being habitual diet. Intestinal microbiota
748 profiling conducted on faecal samples of various cohorts ranging from the Hazda
749 hunter-gatherers of Tanzania to subjects residing in westernised urban areas has
750 revealed how diet shapes the composition and function of the mammalian
751 microbiome. Nutrient products generated by commensal bacterial fermentation in the
752 colon affect fat storage, metabolism and even the gut-brain axis. In **Figure 2** a
753 schematic overview of how extrinsic factors like diet and antibiotics influence
754 normal gut microbiota leading to dysbiosis is presented. Interestingly, the gut
755 microbiota is significantly associated with the rate of ageing of the human organism
756 (Heintz and Mair, 2014).

757 Thus the gut microbiota is a promising target for novel therapeutics for a wide range
758 of conditions and for the manipulation of the progression of ageing itself (Foxx-
759 Orenstein *et al*., 2010; Cani and Delzenne, 2011; Candela *et al*., 2014). Culturomics
760 and phylogeny are indispensable for the development of novel microbial therapeutics
761 as they offer a tool for a more detailed study of bacterial physiology and metabolism
762 (Walker *et al*., 2014). Understanding gut bacterial physiology will also facilitate the
763 development of *in silico* simulation models of the host-microbe / microbe-microbe
764 interactions (Thiele *et al*., 2013).



765

766 **Figure 2 A schematic representation of how extrinsic factors like diet and**
767 **antibiotics influence normal gut microbiota leading to dysbiosis.** The Wiggum
768 plots at the bottom represent normal gut microbiota and gut microbiota in dysbiosis.
769 Coloured circles represent correlated genera and their interactions are schematically
770 indicated by lines. T2D: Type-2 diabetes, NAFLD: non-alcoholic fatty liver disease,
771 NASH: non-alcoholic steatohepatitis, IBS: irritable bowel syndrome, IBD:
772 inflammatory bowel disease.

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1.2 The gut microbiota is a modifiable factor for maintaining health

1.2.1 Introduction

In the previous section I reviwed the factors that shape the human gut microbiome throughout life and I discussed how GIT and extra-intestinal disease, and ageing affect and are affected by the gut microbiota. The colonisation process of the GIT tract begins shortly after birth; the first colonisers are facultative anaerobes originating from vaginal or skin microbiota depending on delivery mode, and are gradually succeeded by anaerobic microorganisms selected by host genetics, diet and the natural environment of the individual, including other people such as family members (Messer *et al*., 2017). The gut microbiota of individuals can be viewed as part of a wider "local metacommunity" due to the microbial exchange that occurs among individuals and with their envoronment (Adair and Douglas, 2017).

Although host-associated factors such as immunity and GIT physiology remain important (Kurilshikov *et al*., 2017), environment is emerging as the most determinant one in the long-term shaping of the microbiome. Recently, Rothschild *et al*. (2018) showed that in spite of some degree of heritability, the gut microbiome is largely shaped by external environmental factors that prevail on host traits.

Studies on the gut microbiota of urban and pre-agricultural modern populations have shown the significant impact of the environment (that is, an array of environmental factors including diet and life-style) on the shaping of the gut microbiota (reviewed in Mancabelli *et al*., 2017). A clear example of environmental impact (available diet, microbial communitites of their habitat) on the microbiota is that of the microbiota (gut, skin, oral) of the highly isolated Yanomami Ameridians (Clemente *et al*., 2015).

Isolation and lack of communication with external microbial communities contributed to the retention of the compositional robustness of the Ameridians microbiome which is not only the richest in diversity and functions so far studied-compared to both Westernised and other non-Westernised cohorts, but also one demonstrating little inter-individual variability (Clemente *et al*., 2015).

As discussed in Chapter 1.1., habitual dietary patterns impact the individual's longitudinal microbiota compositional profile whereas dietary shifts lead to reproducible microbiota responses (Flint *et al*., 2017). For example, O'Keefe *et al*. (2015) showed that a 2-weeks switch of habitual diet between African Americans that were in high CRC risk due to their high-fat/low fibre diet, and low cancer risk rural Africans regularly consuming a low-fat/high-fibre diet, resulted in changes associated with the switched diets in both the gut microbiota composition and metabolome, and in colon mucosa CRC biomarkers.

The association of the gut microbiota and dysbiosis with a plethora of disease that span all aspects of human physiology (reviewed in Chapter 1.1), and the responsiveness of the microbiota to external stimuli such as diet, makes the gut microbiota a promising therapeutic target (Levy *et al*., 2017). Importantly, recent studies on animal models and humans have offered more evidence for the association of dysbiotic microbiota with ageing (Jackson *et al*., 2016; Li *et al*., 2016; Thevaranjan *et al*., 2017) Therefore, gut microbiota modulation is a promising strategy towards ameliorating disease and also improving the life of the elderly in whom gut microbiota compositional and metabolic changes correlate with age-related conditions and co-morbidity (Duncan and Flint, 2013). In the following sections I will review the gut microbiota modulation approaches used to date.

1.2.2 Prebiotics, Probiotics, and next-generation approaches

The term prebiotic was first coined two decades ago and its definition has been repeatedly updated in order to encompass and reflect our increasing understanding of how the interplay between diet and gut microbiota affects host health (Bindels *et al*. 2015). Prebiotics have moved from being a "non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health" described by Gibson and Roberfroid (1995) to a "substrate that is selectively utilized by host microorganisms conferring a health benefit" (Gibson *et al*., 2017). This latest definition of the term prebiotic brought a consensus to the various definition updates of the last 20 years. According to this definition, selectivity is important and not restricted to bifidobacteria and lactobacilli that have been extensively used and studied as the main prebiotic targets (Turroni *et al*., 2014). Importantly, a prebiotic may act on body parts other than the GIT that are niches of microorganisms and microbiota-mediated health effects must be demonstrated in the relevant host (humans or other animals) (Gibson *et al*., 2017). If some evidence of causality is provided between administration and health effect, other non-carbohydrate substrates may qualify as prebiotics (Gibson *et al*., 2017).

Based on the existing scientific evidence, fructo-oligosaccharides (FOS) and galacto-oligosaccharides (GOS) are considered the substrates with most supportive data to fit the description of prebiotics whereas other oligosaccharides (OS) including human and bovine milk OS and other substrates such as dietary fibre, phenolic compounds, conjugated linoleic acid (CLA) and polyunsaturated fatty acids (PUFA) are prebiotic candidates but further research is required in order to confirm the prebiotic activity (Gibson *et al*., 2017). The health benefits of prebiotic administration is likely to be

mediated through microbial metabolite release and homeostatic signalling as a result of the selective prebiotic fermentation by target microorganisms (Gibson *et al*., 2017). The selectivity is relevant to specialised bacterial hydrolases for complex carbohydrates and mechanisms for substrate transfer into the cytoplasm which has been extensively described for the prototypical prebiotic taxa lactobacillus and bifidobacteria and the metabolism of OS (Goh and Klaenhammer, 2015).

Probiotics are organisms that when administered alive and at a certain dose confer a health benefit to the recipient organism (Gareau *et al*., 210). As mentioned before, bifidobacteria and lactobacilli are model probiotics and this can be attributed to certain aspects of their metabolism related to host colonisation and adaptation, such as dietary polysaccharide degradation, host-mucus utilization and bile salt hydrolysis (Turroni *et al*., 2014). Certain physiological aspects such as the ability to survive acidic conditions and bile salt exposure in the GIT after administration, and oxygen tolerance, make the typical probiotics bifidobacteria and lactobacilli technologically manageable, partly explaining why probiotic research and development has focused on these taxa (Duncan and Flint, 2013). Importantly, the dominant notion that probiotic activity is restricted to certain strains within a taxon is evolving: in spite the fact that some strains may not have been thoroughly tested in human trials, minimum probiotic potential is being recognised in these strains due to the fact that they belong in well-established probiotic species and they demonstrate certain species-wide characteristics related to probiotic activity (Sanders *et al*., 2018). Such characteristics are cell-wall structure including mucin-binding proteins, pili and S-layer proteins, exopolysaccharides, complex polysaccharide metabolic pathways, bile salt hydrolases and various primary and secondary metabolites such as SCFAs and AA (reviewed in Lebeer *et al*., 2018; Sanders *et al*., 2018).

Probiotics may contribute to health through various ways. The integrity of the gut barrier is essential for host homeostasis (Köning *et al*., 2016) and administration of probiotic *Lactobacillus* strains has been reported to improve tight junctions and barrier function *in vitro*, in murine models and humans (Karczewski *et al*., 2010; Miyauchi *et al*., 2012; Mujagic *et al*., 2017; Paveljšek *et al*., 2018). The SCFA butyrate is essential for gut barrier function and immunity, as discussed in previous sections, and probiotic administration can contribute to its production in the colon. The metabolites lactate and acetate produced by prototypical probiotics such as strains of bifidobacteria, can serve as substrates in cross-feeding interactions with butyrate producing colonic bacteria (reviewed in Rivière *et al*., 2016). Probiotics can regulate host homeostasis through microbial-associated molecular patterns (MAMPs) recognised by host receptors (e.g. Toll-like receptors) and through secreted molecules triggering immunity host responses like secretion of anti-inflammatory cytokines (van Baarlen *et al*., 2013; Sanders *et al*., 2018). Importantly, these responses vary and are defined by inter-individual genetic differences and the probiotic strain (van Baarlen *et al*., 2013; Sanders *et al*., 2018). Probiotics may contribute to regulation of metabolism (Simon *et al*., 2015) and may improve cognition through eliciting neurotransmitter and anti-inflammatory cytokine release, by being involved in γ-aminobutyric acid (GABA) and tryptophan regulation and in the regulation of the hypothalamic-pituitary-adrenal (HPA) axis among other mechanisms (reviewed in Sarkar *et al*., 2016).

***Next-generation probiotics*:** Lactobacilli and bifidobacteria are the model probiotic organisms that research, regulatory authorities and industry have focused on to date (Duncan and Flint, 2013). Other bacteria such as *E. coli*, *Bacillus* spp., various enterococci, *Weissella* spp. and the fungus *Saccharomyces* have been also used as

probiotics (O'Toole *et al*., 2017). Importantly, next-generation sequencing and metagenomics that have enabled a better understanding of the role of certain GIT bacterial consortia in health and disease (Gilbert *et al*., 2016), and the latest culturomics interest that has enlarged the list of isolated and routinely maintained in the laboratory GIT strict anaerobes (Lagier *et al*., 2015), are contributing towards the development of novel probiotics.

Candidate probiotics can be strictly anaerobic health-relevant taxa that are dominant or subdominant members of the "healthy" gut microbiota, as opposed to the traditionally used probiotics that do not reach dominant abundances in the healthy gut, and have a record of reduced abundance in certain disease (Duncan and Flint, 2013). Next-generation probiotics must comply with the probiotic definition and the traditional regulatory requirements for probiotics, that is, safety record and a well-established pre-clinical and clinical record for the health-benefit conferred to the host by its administration (O'Toole *et al*., 2017). *Bacteroides fragilis* and *B*. *xylanisolvens*, *F*. *prausnitzii*, *A*. *muciniphila*, and *Clostridum butyricum* are candidates for next-generation probiotics (NGPs) (reviewed in O'Toole *et al*., 2017). Importantly, given the effort to develop NGPs based on gut microbiota members for which significantly reduced abundances have a causal link to targeted disease, NGPs or live bacteriotherapeutics, including isolated bacteria or bacterial consortia, may be likely to be regulated and delivered as pharmaceutical products rather than food products as the traditional probiotics (O'Toole *et al*., 2017).

1.2.3 Live bacteriotherapy alternatives to FMT

***Faecal Microbiota Transplantations*:** Faecal microbiota transplantation (FMT) is the gastrointestinal administration of faecal material in suspension from a donor to a

recipient, aiming to confer a health benefit by altering the recipinet's gut microbiota composition and potentially eliminating pathogenic colorization (Gupta *et al*., 2016). Faecal microbiota transplantation has been successfully used to treat *Clostridium difficile* infection (CDI) and it is recommended for clinical practice that FMT be used in relapsing CDI when antibiotic treatment fails and when the risk of morbidity is prevalent (Gupta *et al*., 2016). *Clostridium difficile* exploits the inflammatory state of the "dysbiotic" GIT often but not exclusively related to antibiotics exposure, in order to colonize the colon either asymptomatically or causing diarrhoea of variant severity due to toxin release that confers an advantage to the pathogen against commensals (Hryckowian *et al*., 2017). Importantly, CDI correlates to reduced phylogenetic diversity in the gut microbiota as observed in hospitalized CDI patients (Vincent *et al*., 2013).

Faecal microbiota transplantation introduces GIT commensals that compete for nutrients and niche with pathogens (Kelly *et al*., 2015). Increase in the post-FMT phylogenetic diversity of the recipient's gut microbiota plays a significant role in *C. difficile* colonisation resistance and CDI resolution (Schubert *et al*., 2015; Khanna *et al*., 2017). Increase in the conversion of primary bile salts to secondary and in the abundance of relevant bacterial taxa involved in the bile salts metabolism such as *Clostridium scindens* of the Lachnospiraceae in the recipient faecal microbiota correlates with resolution of CDI (Buffie *et al*., 2015; Staley *et al*., 2016). Introduction of gut microbiota commensals that are able to utilize mucosa-derived sugars like sialic acids is reported as another mechanism of CDI resolution through FMT (Ng *et al*., 2013). Antimicrobials released by members of the "healthy" donor faecal microbiota and the increase in abundance of butyrate-producing bacteria are

other routes through which FMT may ameliorate CDI (Rea *et al*., 2011; Antharam *et al*., 2013).

Given that antibiotic exposure may lead to reduced resilience to pathogens/pathobionts colonisation and expansion in the GIT of taxa such as *C. difficile*, enterococci, *Klebsiella* and *E. coli* (Lewis *et al*., 2015), and the fact that other than *C. difficile* bacteria such as *Clostridium perfringens* and *Staphylococcus aureus* are associated with antibiotic-associated diarrhoea (AAD) (Larcombe *et al*., 2016), FMT could be efficient in treating AAD of various bacterial aetiology. Importantly, it is postulated that the therapeutic potential of FMTs can reach beyond the treatment of AAD and it can be extrapolated in other disease where dysbiosis is strongly associated with the condition. For example, faecal microbiota transplantation has been used to treat UC and CD (Moayyedi *et al*., 2015) and FMT from lean to obese individuals resulted in amelioration of insulin resistance (Vrieze *et al*., 2012; He *et al*., 2017). In spite of the promising results from recent case studies of FMT treatment of conditions like IBD and metabolic disease, and contrary to CDI FMT treatment for which some scientific consensus exists allowing for recommendations and guidelines to clinicians, more research is necessary to establish similar workflow for other conditions such as IBD, IBS and metabolic disease (Cammarota *et al*., 2017).

Faecal microbiota transplantation is highly efficient in the treatment of recurrent CDI and holds some promise for the treatment of other dysbiosis-related conditions. Importantly, adverse outcomes have been reported for FMT including IBD flare and aspiration, and the long-term effect of FMT on the metabolic status of the recipient is yet unknown (Kelly *et al*., 2015). There is scientific interest in refining the FMT

approach in order for its implementation to be more clinician- and patient- friendly, safer and more standardized (Cammarota *et al*., 2017). Importantly, research on how donor strains establish in the recipient gut microbiota environment is pointing towards the development of defined gut microbiota consortia that will allow for a highly personalised therapeutic strategy where recipient GIT ecology and immunity are taken into consideration for the development of the therapeutic consortium (Li *et al*., 2016).

Examples of live bacteriotherapy promoting health: Lawley *et al*. (2012) demonstrated in a murine model of recurrent CDI that a cocktail of six faecal bacteria (MixB) isolated from healthy mice was as efficient in resolving infection as FMT from healthy to diseased mice. The MixB was composed of the taxa *Staphylococcus warneri*, *Enterococcus hirae*, *Lactobacillus reuteri* and the newly then isolated *Anaerostipes* sp., *Bacteroides* sp. and *Enterorhabdus* sp. that were isolated from faecal samples used for the comparison FMT. The study underlined the importance of the phylogenetic diversity of the bacterial combination in resolving CDI and restoring the murine faecal microbiota to pre-infection composition and further showed that a mix of *Bacteroides*/*Lactobacillus* representing a probiotic-based treatment was not able to restore dysbiosis and resolve infection. Using a murine models Atarashi *et al.* (2013) isolated 17 strains from a human faecal microbiota donor that were able to induce Treg cell proliferation and anti-inflammatory components when introduce in germ-free mice. These strains that belonged to *Clostridium* clusters XIVa, IV and XVIII were efficient in attenuating inflammation in a chemically induced colitis murine model (Atarashi *et al*., 2013).

Petrof *et al*. (2013) introduced the "RePOOPulate" stool substitute that was used to treat refractory CDI in two patients with a highly infectious *C*. *difficile* strain. The bacterial mix consisted of 33 strains isolated from the faecal sample of a healthy adult and belonged to various phylogenetic groups that are abundant in the gut microbiota (Petrof *et al*., 2013). Six months after the administration of "RePOOPulate" stool the patients were free of CDI symptoms and sequences (as revealed by 16S rRNA gene sequencing) not abundant pre-treatment were still present post-treatment suggesting a perseverance of the introduced bacteria in the recipient microbiota (Petrof *et al*., 2013). This consortium referred to as microbial ecosystem therapeutic-1 (MET-1) was subsequently used in a murine model of *Salmonella typhimurium* infection (Martz *et al*., 2015). MET-1 could not resolve infection but was found to be protective of tight-junctions activity which prevented the pathogen from establishing systemic infection (Martz *et al*., 2015). The microbial ecosystem therapeutic-2 (MET-2) was developed from 33 strains isolated from the faeces of a healthy donor representing the minimal gut microbiota of the derivative donor (Yen *et al*., 2015) and is currently being used in a clinical trial for the resolution of refractory CDI (https://clinicaltrials.gov/ct2/show/study/NCT02865616). In another recent clinical trial, Khanna *et al*. (2016) successfully treated CDI patients with SER-109, a therapeutic mix of encapsulated spores from 50 spore-forming Firmicutes species isolated from healthy donors.

1.2.4 *In vitro* and *in vivo* models for the development of gut microbiota therapeutics

In vitro GIT models and *in vivo* animal models are facilitating research on the therapeutic manipulation of the gut microbiota before applying these therapeutic approaches in clinical trials.

***In vitro* models:** Single- and multi-stage chemostats have been used for decades for temperature and pH controlled anaerobic faecal fermentations of selected substrates allowing for the simulation and study of basic aspects of faecal microbiota metabolic activity (Allison *et al*., 1989; Macfarlane *et al*., 1989). Model design varies from a single stage chemostat operating as batch or continuously, to multistage interconnected chemostats that operate in a continuous flow, simulating one or various parts from proximal to distal colon depending on the set pH of the different vessels (Payne *et al*., 2012). More advanced continuous models such as SHIME and TIM aim at incorporating the host digestion factor (Payne *et al*., 2012). TIM-2 was developed based on the stomach and small intestine TNO TIM-1 *in vitro* GIT model, and is a computer-controlled model for the large intestine that can simulate peristaltic movements and dialysis (reviewed by Venema, K., 2015). The simulator of the human intestinal microbial ecosystem SHIME is composed of five vessels mimicking the GIT from the stomach to the colon parts (reviewed by Van de Wiele *et al*., 2015). Other *in vitro* models that are being used are the 4-vessel simulator of colon parts EnteroMix and the Lacroix model that allows for better growth of both lumen-living and attached to surfaces microbiota (reviewed in Venema and Van den Abbeele, 2013).

In vitro models present inherent limitations such as the lack of the host immune and metabolic responses, the lack of epithelial cell barriers and mucin adhesion sites, and

the lack of the high level of compartmentalisation that is achieved in the host GIT (Macfarlane and Macfarlane, 2007). Researchers have developed various *in vitro* model set-ups aiming at tackling these limitations; for example the multistage systems that have been developed in order to simulate various GIT tract parts as discussed previously. The introduction of mucin surfaces in two-stage systems offered an attachment site for sessile faecal bacteria such as *Bacteroides fragilis*-related taxa, clostridia and enterobacteria (Macfarlane *et al*., 2005). Cinquin *et al*. (2006) applied a faecal microbiota immobilization technique in order to prevent wash-out of faecal microbiota in a three-stage continuous system. Modifications of the SHIME system allowed for incorporating a mucin environment (M-SHIME) that deterred wash-out of sessile members of the faecal microbiota such as *Clostridium* cluster XIVa belonging taxa leading to more *in-vivo* relevant conditions (Van den Abbeele *et al*., 2012; Van den Abbeele *et al*., 2013). In a more sophisticated set-up, SHIME was combined with a platform of host cells and microbiota interaction (HMI) (Marzorati *et al*., 2014).

In spite of the limitations, *in vitro* systems allow for a relatively low-cost, straightforward and high-throughput approach to study the effect of various substrates from candidate probiotics and prebiotics to novel drugs on the compositional and metabolic profile of the faecal microbiota deriving from healthy or diseased donors (Macfarlane and Macfarlane, 2007). The compositional effect on the faecal microbiota is analysed with various molecular methods such as 16S rRNA gene next-generation sequencing, qPCR and fluorescent *in situ* hybridisation (FISH) and analytical techniques such as HPLC are being used for SCFA production.

For example, Van den Abbeele *et al*. (2013) compared the effect of long-chain arabinoxylan (LC-AX) and inulin on the human faecal microbiota in SHIME and TIM and reported that in both systems LC-AX demonstrated similar to inulin prebiotic effects on distinct bifidobacteria population and SCFA production. Compared to the FOS faecal fermentation pattern that was utilized in the simulated proximal colon area, arabinogalactan (AG) was available for fermentation in distal parts of the simulated colon in the SHIME system revealing a potential role of AG in reducing the distal colonic pH by increasing carbohydrate fermentation in the distal colon and promoting the growth of butyrate-producers such as *F. prausnitzii* (Terpend *et al*., 2013).

Chung *et al*. (2016) using a three-vessel *in vitro* system with variant fixed-pH conditions showed that the prebiotic effect of non-digestible substrates like pectin and inulin is determined by the faecal microbiota inter-individual compositional variations, the fluctuations in pH that naturally occur in the colon as opposed to the stable conditions of the simulated colonic fermentations, and that responses to substrates cannot be easily extrapolated to the whole taxonomic structure based on individual taxon-members responsiveness. Poeker *et al*. (2018) investigated the prebiotic effect of the dietary fibres β-glucan, a-galacto-oligosaccharides (α-GOS) and xylo-oligosaccharides (XOS) on the faecal microbiota of healthy individuals using a three-vessel continuous system (PolyFermS). They reported that the *in vitro* response of the faecal microbiota was largely dependent on the inter-individual variations of the baseline microbiota and proposed that the metabolic responses of the microbiota such as the SCFA production can be the most comparable measure of the prebiotic potential of test substrates on microbiotas from different subjects (Poeker *et al*., 2018).

Other potentially prebiotic substrates tested in *in vitro* systems are wheat dextrin, α-gluco-oligosaccharides, fructo-, galacto- and iso- malto- oligosaccharides, lactulose and lactosucrose (Hobden *et al*., 2013; Sarbini *et al*., 2013; Takagi *et al*., 2016), as well as food ingredients such as whey, fruits and phenolic compounds (Sanchez-Patan *et al*., 2015; Koutsos *et al*. 2017; Sanchez-Moya *et al*., 2017). Probiotics have been tested in *in vitro* systems alone or combined with prebiotic substrates and in combination with pathobionts in pre-clinical tests investigating the potential of probiotics to prevent pathogen establishment in the GIT tract (Martinez *et al*., 2013; Cardarelli *et al*., 2016; van den Abbeele *et al*., 2016).

In vitro systems simulating colonic fermentation in studies of prebiotic and probiotic modulation of the faecal microbiota of healthy older subjects have been used but to a limited extent (Likotrafiti *et al*., 2014; Liu *et al*., 2016). Fehlbaum *et al*. (2015) proposed the use of the adjusted PolyFermS multi-stage *in vitro* system as a reproducible set-up for the study of various factors such as diet, on the faecal microbiota of older donors. The PolyFermS employed immobilized faecal microbiota to continuously seed connected fermentation vessels and medium turn-over period was regulated in order to represent colonic transit times observed in healthy older people (Fehlbaum *et al*. 2015). Faecal microbiota from healthy elderly has been used in *in vitro* studies and to my knowledge no study has explored the *in vitro* modulatory effect of prebiotic candidates on the low-diversity faecal microbiota from frail elderly.

Murine models in gut microbiota research: Mouse models such as germ-free (GF) and gnotobiotic mice, human microbiota associated (HMA) and conventional mice, offer an *in vivo* environment to study interactions between the gut microbiota and

exogenous factors like diet and therefore, circumvent to some extent the aforementioned limitations of the *in vitro* environment. In spite of caveats in translating observations from mice to humans, murine models have largely facilitated pre-clinical research on the host-microbiota interactions and causality with regards to health and disease, diet and novel therapeutics (Nguyen *et al*., 2015).

Germ-free (GF) mice are reared aseptically in order to prevent the development of their indigenous gut microbiota, they are kept under sterile conditions throughout their life and they allow the researcher to define the gut microbiota of the experimental animal by selected colonisation (Martin *et al*., 2016). Conventionalisation can be performed by transplanting selected microbiota strains or whole faecal microbiota from healthy or diseased donors, mice or humans ("humanisation"). Due to shared host responses and colonisation mechanisms, microbiotas from ecologically divergent hosts can survive and establish in other than their indigenous GIT niche (Rawls *et al*., 2006; Seedorf *et al*., 2014).

The primary concern with gnotobiotic models is the successful establishment of the donor microbiota and the potential metabolic impact on the recipient animal. Bäckhed *et al*. (2004) using GF C57BL/6 adult mice reported that microbiota transplantation from conventional to GF mice resulted in increased energy harvest mediated by the transplanted microbiota showing not only the importance of the gut microbiota in energy harvesting for the host but also that the transplantation could alter the recipient metabolic profile. Turnbaugh *et al*. (2006) demonstrated that metabolic profiles like obesity that are associated with certain gut microbiota composition (Ley *et al*., 2005) can be transmitted from conventional obese mice to GF. The potential of metabolic profile transfer through conventionalisation is an

essential tool in metabolic disease and obesity pre-clinical research allowing for causality and mechanistic observations on how the whole microbiota composition, certain taxa and the metabolome respond to Western diet mediating adiposity (Turnbaugh *et al*., 2009).

Due to rearing in the absence of the indigenous microbiota, the GF mouse has certain GIT physiology alterations and immunity impairments compared to conventional animals such as fewer and smaller Peyer patches and mesenteric lymph nodes, alterations in the villus formations of the epithelial cells and lower expression levels of Toll-like receptors (reviewed in Round and Mazmanian, 2009). These physiological differences compared to conventional animals and constraints regarding the laborious process of generation and maintenance of GF murine strains, have led to the development of methods for the depletion of the indigenous gut microbiota of conventional mice with antibiotic (Abx) treatment (Bereswill *et al*., 2011; Hintze *et al*., 2014). The metabolic profile transfer demonstrated in GF murine models was partly demonstrated in ampicillin-treated C57BL/6 adult and weaning mice that received microbiota transplants from obese and lean mice (Ellekilde *et al*., 2015). The donors microbiota compositional profile was maintained and some obesity-related clinical outcomes were observed for a certain post-transplantation period before the microbiota composition begun to revert back to the murine baseline (Ellekilde *et al*., 2015).

Antibiotic treatment of conventional mice prior to microbiota transplantation presents certain advantages and disadvantages compared to GF mice (summarised in Lundberg *et al*., 2016). The relatively lower maintenance cost and the fact that the Abx approach can be applied to the desired mouse strain by the researcher as

opposed to the limited available GF mouse strains that are prepared in specialised laboratories are some advantages of the Abx method. The fully mature immune system of the Abx mice is another comparative advantage. Overgrowth of pathobionts, antibiotic resistance expansion and re-emergence of the indigenous baseline microbiota are some of the negative aspects of the Abx approach that may lead to spurious reproducibility. Furthermore, it has been reported that conventionalisation of GF C57BL/6 adult mice resulted in "conventionalisation" of the innate and adaptive immune system 30 days post microbiota transplantation (El Aidy *et al*., 2012; El Aidy *et al*., 2014). Importantly, Chung *et al*. (2013) demonstrated that it is the mouse-related (or host-related) gut microbiota that can "normalise" the immature GF mouse immunity. The potential of achieving normalisation of the immune system of GF mice within a certain time should be taken into account when designing experiments on host-immunity-microbiota interactions.

***Efficacy of human microbiota colonisation of the murine gut and use of conventional mice in gut microbiota studies*:** Another point of consideration on gnotobiotic mouse models is the efficacy of the establishment of the xenomicrobiota in the recipient gut. It is well documented that the host selective pressure that shapes the indigenous microbiota will be also applied to the transplanted microbiota reshaping it in order to match the host physiology, immunity and diet (Messer *et al*., 2016). Therefore, certain donor species such as taxa that depend on host-specific niches like the mucosa, may be proven unable to establish in the recipient gut rendering the transplanted microbiota significantly dependant on the given diet (Arrieta *et al*., 2016). Wos-Oxley *et al*. (2012) comparing the colonisation patterns of GF rats, GF mice and ciprofloxacin-treated mice with human faecal microbiota

under a low-fat high-polysaccharide diet, reported that mice favoured the Bacteroidetes establishment whereas major Firmicutes taxa belonging to *Clostridium* cluster IV, XIVa and the *Clostridium* leptum group did not reach a high level of colonisation efficacy.

Arrieta *et al.* (2016) proposed that, at least in studies focusing on the overall ecological effect on the gut microbiota of an exogenous factor such as diet, conventional mice with their intact gut microbiota could be a more informative pre-clinical approach. Caveats in the extrapolation of conclusions from studies with conventional mice to humans that to some extent can be generalised for the aforementioned murine models, are the differences in physiology and anatomy of the murine and human GIT tract including size, mucosal surfaces, the existence or not of forestomach and the caecum size, that define specific niches for host-adapted taxa (Nguyen *et al*., 2015). The taxa shared between murine and human gut microbiota significantly differ in relative abundances and distinct bacterial groups dominate the two microbiotas; for example, *Faecalibacterium*, *Ruminococcus* and *Prevotella* are dominant in the human gut microbiota whereas *Lactobacillus*, *Alistipes* and *Turicibacter* dominate the murine gut (Nguyen *et al*., 2015).

Importantly, the murine microbiota differs between the various available mouse strains and even within strains, the provider company, the maintenance facilities, co-housing and coprophagy may significantly contribute to the baseline murine microbiota, thus affecting reproducibility of experiments and comparisons between studies (Hugenholtz and de Vos, 2018). Given the importance of mouse models in host-gut microbiota research, the determinative role of the baseline murine microbiota in the study outcomes and the lack of extensive studies focusing on the

indigenous murine microbiota, Lagkouvardos *et al*. (2016) established a collection of isolated murine bacterial strains, analogous to human culturomics.

So far, I have discussed major mouse models i.e. GF and Abx-treated mice conventionalised with murine microbiota or "humanised" with human microbiota, and points of consideration for each approach such as immune responses in GF mice and efficacy of colonization with xenomicrobiota. Importantly, conventional mice are a valuable tool in the study of host-microbiota interactions since every model is accompanied by certain considerations when it comes to translating observations from mouse to humans. With regards to reproducibility, gnotobiotic mice remain a well-controlled, reproducible animal model used in many studies that have significantly contributed to our increased understanding of the complicated interactions between gut microbiota-host-dysbiosis-exogenous factors. The selected *in vivo* studies shown in **Table 3** demonstrate the breadth of the scientific questions conserning gut microbiota diet, health and disease that have been investigated through employing mouse models and it is not an exhaustive review of the existing murine model studies.

1843 **Table 3 Selected studies employing murine models in the study of gut microbiota, diet, health and disease.**

Mouse model	Donor microbiota	Condition	Test substrate	Outcomes	Reference
C57BL6/J GF	Human	Effect of diet on the microbiome	Low-fat polysaccharide rich diet High-fat sugar rich "Western" diet	– human faecal microbiota establishment in GF mice – microbiota transmission from gnotobiotic mice to offspring – rapid and reproducible responses in composition metabolome and gene expression of the microbiota due to switch to "Western" diet altered – transmission of host diet-induced adiposity to gnotobiotic mice – isolation of obesity associated Erysipelotrichia taxa	Turnbaugh *et al*. 2009
C57BL6/J conventional	-	Diet-induced obesity	High-fat diet (HF) Arabinoxylan (AX)	AX restored gut microbiota changes due to HF diet: – ↑ *Bacteroides-Prevotella* spp., *Roseburia* spp. – ↑ caecal bifidobacteria AX decreased adiposity, improved tight junctions function	Neyrinck *et al*., 2011
C57BL6/J conventional	-	Diet-induced obesity	High-fat diet (HF) Arabinoxylan OS (AXOS)	AXOS restored obesity measurements: – ↓ insulin resistance – ↓ metabolic endotoxemia – ↓ macrophage infiltration – ↓ IL6 in plasma AXOS improved gut microbiota factors: – ↑ caecal bifidobacteria – Up-regulations of tight junction proteins	Neyrinck *et al*., 2012

Mouse model	Donor microbiota	Condition	Test substrate	Outcomes	Reference
Swiss-Webster C57BL6/J GF conventional	Various human donors *B. thetaiotaomicron* mono-colonisation *B. thetaiotaomicron and B. longum bi-colonisation*	"humanisation" and diet impact on faecal and urinary metabolome	Polysaccharide-rich diet (PD) Polysaccharide-deficient diet (PDD)	– distinct metabolomes in humanised and conventional mice – distinct clustering of the "humanised" microbiotas (UniFrac distances) and metabolomes based on PD and PDD diet – colonisation with simple defined microbiotas model to some extent the "humanisation" metabolic process	Marcobal *et al*., 2013
C3H/HeOuJ GF	8 bacterial strains from human gut microbiota	diet-induced obesity	+/-*Clostridium ramosum* (Erysipelotrichia) High-fat diet (HFD) Low-fat diet (LFD)	– HFD promoted *C. ramosum* abundance and obesity – *C. ramosum* related transcription upregulation of glucose and fat transport proteins in jejunal and ileum mucosa	Woting *et al*., 2014
C57BL6/J conventional	-	Frailty and ageing	Standard chow	frailty index correlated to ageing characteristics of the "aged" murine microbiota composition and function : – ↑*Alistipes* (Rikenellaceae) – ↓cobalamin, biotin biosynthesis – ↑creatine degradation related to muscle wasting – ↓di-,oligo- and poly-saccharide utilisation genes	Langille *et al.*, 2014
C57BL6/J GF	Human	*C. difficile* infection	Antibiotic treatment *C. difficile* spores	– "humanised" microbiota transferred to offsprings – establishment of CDI and recurrent CDI in gnotobiotic murine model	Collins *et al*., 2015
C57BL6/J /RccHsd conventional GF	Murine	ageing and systemic inflammation	Standard chow	microbiota transfer from conventional old to GF young mice promoted inflammageing : – ↑TM7 and Protoebacteria taxa – ↓Akkermansia – ↑ T cell activation – ↑inflammation inducing bacterial components in circulation	*Fransen* et al., 2017

1.3 The effect of milk and glycomacropeptide on the gut microbiota

1.3.1 The effect of milk on human health and the gut microbiota

Dairy products and milk have been a component of human nutrition since the seventh millennium BC (Evershed *et al*., 2008). According to the Food and Agriculture Organisation of the United Nations (FAO) (http://www.fao.org/home/en/) in Europe the annual consumption of milk rises to more than 150 kg per capita providing to consumers 9% of the dietary energy supply, 19% of the protein supply and the 11%-14% of the dietary fat. Milk consumption is higher in developed countries but many developing countries are steadily increasing their per capita milk consumption (FAO; Gateway to dairy production and products).

Milk is a rich source of nutrients (**Table 4**) composed of lipids including saturated and unsaturated fatty acids, milk fat globule membranes (MFGM), phospholipids and glycosphingolipids, proteins such as casein and whey, vitamins such as vitamin A, E and B12, and minerals including calcium, magnesium, phosphorus and zinc (Haug *et al*., 2007). The carbohydrate profile of bovine milk is composed mainly of the disaccharide lactose, traces of neutral and acidic sialic acid containing oligosaccharides, and glycoconjugates (Gopal and Gill, 2000; Hsieh *et al*., 2015). Glycoproteins like the extensively sialylated mucins, and glycolipids comprise the glycoconjugate component of bovine milk (O'Riordan *et al*., 2014).

The beneficial effect of milk and dairy in human diet has been challenged mostly on the basis of the high saturated fat content in milk but the evidence supporting this notion is inconclusive. Meta-analysis of various observational and prospective cohort studies has concluded that no consistent association between milk consumption and

increased all-cause mortality can be established (Guo *et al*., 2017). Conversely, various studies including epidemiological studies indicate the multitude of health benefits of moderate milk consumption (Visioli and Strata, 2014).

Table 4 Bovine milk components. The data is taken from the Society of Dairy Technology (https://www.sdt.org/pages/) and O'Riordan *et al*. (2014). The table is not exhaustively presenting all milk components, but the main ones.

Component and proportion in bovine milk		Detailed composition
Fat	4%	Small globules covered by the milk fat globule membrane (MFGM), rich in proteins and phospholipids
Proteins	3.3%	• Caseins (76%) in casein micelles structure: α_{s1}, α_{s2}, β, κ • Whey (18%): β-lactoglobulin, α-lactalbumin
Carbohydrates	5%	• Lactose (4.7%) • Free oligosaccharides, glycoconjugates
Minerals	0.7%	Calcium, phosphorus
Organic acids	0.2%	Citric and lactic acid
Vitamins	traces	• Fat-soluble: A, D, E, K • Water-soluble: B_1, B_2, B_6, B_{12}
Water	87%	

1.3.1.1 Cardio-metabolic disease and all-cause mortality

Certain co-occurring metabolic conditions including obesity, insulin resistance and high lipidaemia, grouped under the term Metabolic Syndrome (MetS), may be risk factors for cardiovascular disease (CVD), diabetes type 2 (T2D) and hepatic steatosis

(reviewed in Lusis *et al*., 2008; Catrysse and van Loo, 2017). Cardio-metabolic conditions are prevalent in Western countries; chronic low-grade inflammation is an underlying factor of these conditions and apart from human genetics, high-fat dietary choices and sedentary life style are strongly associated with MetS development (Catrysse and van Loo, 2017).

Evidence from large Western cohorts and meta-analysis of prospective cohort studies support milk and dairy inclusion in a balanced diet and the positive association of increased dairy consumption with a reduction in risk of coronary health and MetS risk (Panagiotakos *et al*., 2010; Benatar *et al*., 2014; Crichton and Alkewri, 2014; Drehmer *et al*., 2016). In Asian countries like Korea and Japan, where in spite of Western influence dietary patterns remain different from those in Western countries, a higher milk and dairy consumption was associated with a lower risk of MetS development (Jun *et al*., 2016). In another non-Western cohort of Iranian adults, regular weekly whole milk consumption but not daily consumption was associated with reduced CVD risk (Talaei *et al*., 2017).

Conversely, in two cohorts (n≈100,000 older adults) in Sweden, where consumption of fluid milk is one of the highest worldwide, a high milk consumption was associated with adverse health outcomes, i.e. all-cause mortality and hip fracture (Michaelsson *et al*., 2014; Tognon *et al*., 2017). Michaelsson *et al*. (2014) observed that ≥3 glasses of milk per day was associated with increased mortality and hip-fracture in two Swedish cohorts followed for 11 and 20 years. Tognon *et al*. (2017) observed that high consumption of non-fermented milk (>2.5 intakes per day) correlated with an increased risk of all-cause mortality in a dose-response manner in a mean follow-up time of approximately 14 y. Importantly, Tognon *et al*. (2017)

observed that non-fermented milk consumption was associated with consumers with poorer educational level compared to cheese consumers who had comparatively reduced all-cause mortality risk. This observation may indicate that residual confounding factors associated with life-style may have masked a more relevant to milk consumption effect on the participants' health.

Milk-based diets were shown to attenuate oxidative stress in obese and overweight subjects compared to non-dairy intervention control diets (Zemel *et al*., 2010; Stancliffe *et al*., 2011). Milk and dairy consumption may improve blood pressure and arterial plasticity potentially due to milk proteins and peptides released upon digestion (Lovegrove and Hobbs, 2016). In spite of the heavy load in whole milk and dairy products of saturated fatty acids (SFA) (Mansson, 2008) evidence suggests that whole dairy (excluding butter) does not contribute to significant or detrimental increase in blood low-density lipoprotein cholesterol (LDL-C) (reviewed in Lovegrove and Hobbs, 2016). For the milk proteins whey and casein, data from clinical studies are relatively consistent indicating towards a potential positive effect on blood glucose regulation (Fekete *et al*., 2016). Importantly, frequent low-fat milk and dairy consumption may be associated with a lower risk of T2D (Aune *et al*., 2013; Díaz-López *et al*., 2016).

Observational studies have often provoked controversy on the effect of milk consumption and all-cause mortality (Larsson *et al*., 2015). Importantly, adjustment for a wide array of confounding factors such as background dietary patterns, life-long exposure to dairy, life-style and exercise, smoking, age, sex, education, self-reported milk and dairy servings could leverage bias in observational study design. Leverage of the confounding factors effect and clinical trials with well-defined dairy

products (ex. non-fermented milk with variant fat content) could further elucidate existing controversy.

1.3.1.2 Frailty

Frailty is a condition of functional disability observed in older age, that is characterised by the gradual loss of homeostatic maintenance of the organism even under minor challenges and it may lead to increased dependency, adverse outcomes after hospitalization and even death (Clegg *et al*., 2013). The frailty phenotype is not restricted to older age but it is also observed in people with advanced HIV and cancer (Deeks, 2011; Ethun *et al*., 2017). Sarcopenia which is the state of de-regulated muscular homeostasis leading to the progressive loss of muscle mass and functionality decline is widely recognised as a syndrome and it is one of the main frailty manifestations; metabolic, inflammatory and dietary challenges may contribute to the development and progression of the syndrome (Fielding *et al*., 2011). Importantly, frailty assessment models can successfully predict the risk of osteoporosis-related bone fracture in older age (Li *et al*., 2017). New drug development, physical activity and dietary interventions are being employed towards reversing the frailty scores in sufferers (Keevil and Romero-Ortuno, 2015).

Muscle maintenance results from the balance between muscle protein synthesis (MPS) triggered by dietary anabolic stimuli like proteins and amino acids and the naturally occurring process of proteolysis (Mitchell *et al*., 2016). Glucose regulation and muscle protein turn-over pass through the homeostatic metabolic junction of the mechanistic target of rapamycin (mTOR) where the essential amino acids especially leucine, and insulin are key signalling molecules (Barzilai *et al*., 2012; Saxton and Sabatini, 2017). In older age anabolic resistance blunts the sensitivity of the

organism to respond to these signals and consecutively to translate the signals to muscle accretion eventually leading to wasting; nutritional interventions with leucine-rich and easily ingested high in protein dietary supplements and physical activity may reverse the adverse outcome of severe muscle loss (Moore, 2014).

Whey protein is considered "fast" in releasing amino acids in blood after digestion and may stimulate postprandial MPS whereas casein that is considered "slow" confers a moderate prolonged aminoacidaemia and supresses muscle protein breakdown (MPB) (Boirie *et al*., 1997). Fast postprandial aminoacidaemia was initially considered as an MPS driver but it seems that the amino acid composition of the dietary proteins ingested (ex. leucine content) is key to the anabolic activity (Mitchell *et al*., 2016). The milk proteins whey and casein that contain all essential amino acids and are rich in leucine (Hall *et al*., 2003) have been studied in human trials in the concept of "fast" and "slow" protein and MPS.

Consumption of fluid skim milk that combines the two types of "slow" and "fast" proteins after exercise resulted in greater muscle accrual in young adults compared to isonitrogenous and isoenergetic soy beverage consumption (Hartman *et al*., 2007; Wilkinson *et al*., 2007). Whey supplementation alone was reported efficient to better sustain protein synthesis and lean mass gain in adults after resistance exercise and compared to casein or soy protein (Tang *et al*., 2009; Volek *et al*., 2013).

For older subjects in whom high quality dietary amino acids intake is essential for muscle accretion, the rapidly digested rich-in-leucine whey protein may be a suitable dietary protein source (Landi *et al*., 2016). Twenty g of whey protein were more effective in postprandial muscle protein retention in healthy men over 74 yrs without any exercise intervention compared to the ingestion of the same amount of casein or

casein hydrolysate (Pennings *et al*., 2011). Mitchell *et al*., (2015) reported that 20 g of either milk protein isolate or whey protein dietary supplementation resulted in the same postprandial muscle protein fractional synthetic rates (FSR) in healthy mostly sedentary men aged 45-60 yrs. This suggests that milk protein is a widely accessible food component and is thus adequate source of dietary protein for older adults.

In observational studies of large population cohorts, older people that are regular consumers of dairy i.e., milk, yoghurt and cheese, have improved measurements for skeletal muscle and bone mass compared to less regular consumers. Observations from the Quebec NuAge cohort of people over 67 yrs, showed that community-residing people consuming ≥2 serving of dairy (milk, yoghurt, cheese) per day had better functionality and mobility measurements and increased lean mass compared to people consuming less servings per day (Farsijani *et al*., 2017). In the Quebec NuAge study the reduced risk of pre-frailty or frailty was more pronounced among men with high dairy intake compared to low intake than in the respective group of women. Among 564 community-dwelling women with mean age 85 yrs, high dairy consumption (≥2.2 servings per day) assessed for the previous 12 months associated with higher bone density and skeletal muscle mass (Radavelli-Bagatini *et al*., 2014). Radavelli-Bagatini *et al*. (2013) had previously reported similar positive effects on muscular and bone health for a larger cohort of women aged 70-85 yrs. In a cohort (n=1,871) of non-hospitalized non-frail adults over 60 yrs, consumption of ≥7 serving per week of low-fat milk and yoghurt was associated with reduced frailty risk in a 3.5 years follow-up (Lana *et al*., 2015).

Regarding bone density and risk of fracture in older age, Sahni *et al*. (2014) observed that for adults over 77 yrs (Farmingham, USA) who regularly consumed milk or

milk and yoghurt, the risk of hip fracture was significantly lower compared to that low in frequency consumers of dairy products. The group also showed that high milk, yoghurt and dairy consumption may benefit bone health among elderly under dietary vitamin D supplementation (Sahni *et al*., 2017a). Recent studies suggest that diets rich in dairy may be beneficial for bone mineral density and reduced risk of hip fracture in elderly population (de Jonge *et al*., 2017; Durosier-Izart *et al*., 2017).

Other studies contradict the beneficial effect or report no effect of milk on skeletal health. For example, a 2011 meta-analysis of cohort studies showed that there was no association between milk consumption and bone fracture risk for older women whereas for men there was a marginal inverse association (Bischoff-Ferrari *et al*., 2011). In two large Swedish cohorts (≥45.000 subjects each) of adults from 39 to 79 yrs old and for a long follow-up period of over a decade high milk intake was positively correlated with higher mortality and hip fracture (Michaelsson *et al*., 2014) as discussed above in this review. The study of Michaelsson *et al*. (2014) reported that the female high-milk-consuming participants showed increased health risk in a 20 yrs follow-up period. This prolonged follow-up period potentially increased the confounding factors; in older adults mortality will increase over the years and older age co-morbidities may have masked the effect of dairy products on the participants' health (Sahni *et al*., 2017b).

1.3.1.3 Other potential health benefits of milk consumption

There are conflicting studies on the beneficial effect of milk consumption on cognition. In older adult populations with Western life-style, total low fat dairy and cheese consumption but not milk alone was reported beneficial for various cognition measurements (Crichton *et al*, 2010; Park and Fulgoni, 2013). Camfield *et al.* (2011)

after a thorough review of existing cross-section studies concluded that low-fat dairy products including milk incorporated in balanced diet may positively affect cognitive function of older adults. Similarly, Wu and Sun (2016) after reviewing available studies on milk and dairy consumption, concluded that there may be a trend suggesting that regular milk consumption improves cognition. This trend was more pronounced in Asian cohorts and it was attributed to the fact that total dairy intake is less common as a background diet in Asia compared to Western countries (Wu and Sun, 2016) making the effect of milk more detectable. More prospective studies with standardized measurements and adjustment for a wider range of cofounding factors (Wu and Sun, 2016) as well as clinical trials are needed in order to draw robust conclusions on the effect of milk on cognitive function.

Many studies have explored the impact of regular milk consumption on several cancers but firm conclusion cannot be drawn yet (Visoli and Strata, 2014). An area of particular interest has been colorectal cancer (CRC) and dairy calcium intake. According to the WHO, CRC is the second most prevalent cause of cancer death among Europeans and a major cause of mortality worldwide with life-style factors such as diet being one of the risk factors (http://www.euro.who.int/en/health-topics/noncommunicable-diseases/cancer/news/news/2012/2/early-detection-of-common-cancers/colorectal-cancer).

Park e*t al*. (2009) conducted a large prospective study on American older adults ≥50 yrs (293,907 men and 198,903 women) and assessed incidents of cancer and dairy products (milk, yoghurt, cheese) consumption over 7 years. The group found no adverse outcomes from dairy consumption and cancer incident; high dairy and supplemental calcium intake was related to 16% and 23% lower risk of GIT cancers

(especially CRC) in men and women respectively. Of note was the observation that people with higher dairy consumption tended to have a healthier life-style and be college educated. Aune *et al*. (2012) conducted a meta-analysis on 19 cohort studies performed until May 2010 and concluded that high milk and total dairy intake (200 g and 400 g per day respectively) was associated with reduced CRC incident.

More recently, Murphy *et al*. (2013) investigated the effect of dairy product habitual consumption and colorectal cancer risk in a prospective study on European adults (EPIC cohort: 10 countries, n=477,122, over 35 yrs old, 11 years follow-up). A portion of whole-fat or skimmed milk and dairy per day but not supplemental calcium was inversely associated with CRC occurrence. In a USA cohort of 77,712 older adults, a 7 year old follow-up study showed that milk consumption and dairy (milk, yoghurt, cheese) may be protective for rectal and CRC; the group suggested that dietary calcium may be protective against CRC (Tantamango-Bartley *et al*., 2017).

The existing data do not suggest a significant effect of milk intake on breast cancer occurrence. A prospective study of 64,904 Norwegian women showed that dairy consumption had no significant impact on pre- or post-menopausal breast cancer incidence (Hjartaker *et al*., 2010). In a meta-analysis of 18 prospective cohort studies intake level of total dairy products (milk, yoghurt, cheese, butter) but not milk alone was associated with reduced risk of breast cancer (Dong *et al*., 2011). Similarly, lung cancer did not seem to be significantly associated to milk or dairy products consumption (Yang *et al*., 2016).

Milk consumption may play a detrimental role in the progression of prostate cancer. Among 3,918 mostly Caucasian men in U.S.A. (Health Professionals Follow-Up

Study) diagnosed with prostate cancer at baseline those that frequently consumed whole milk had increased risk of disease progression compared to the less frequent whole milk consumers or those frequently consuming low-fat dairy products who demonstrated reduced risk for disease progression after 7.6 years of follow-up (Pettersson *et al*., 2013). In another cohort of 926 men diagnosed with non-metastatic prostate cancer (Physician's Health Study) 3 servings of dairy per day associated with higher risk of total and prostate cancer-related mortality compared to men consuming 1 serving per day (Yang *et al*., 2016). Downer *et al*. (2017) reported that ≥3 servings per day of high-fat milk correlated with higher risk of prostate cancer mortality compared to 1 serving per day. Both Yang *et al*. and Downer *et al*. observed a borderline positive association for low-fat dairy and reduced cancer mortality. Some studies have suggested a link between milk and dairy lactose to risk for ovarian cancer (Larsson *et al.*, 2006; Qin *et al*., 2016) whereas dietary calcium was inversely associated to the disease (Qin *et al*., 2016; Song *et al*. 2017).

A small number of recent studies suggest a beneficial role of dairy products in renal health. Regular low fat dairy products and dietary calcium was reported beneficial for kidney function in a cohort of ≥50 yrs old Australians (Gopinath *et al*., 2016). Rebholz *et al*. (2016) also observed a protective effect of a healthy diet rich in low-fat milk and yoghurt against kidney disease in a large cohort of middle-aged African-American and Caucasians.

1.3.1.4 Strengths and weaknesses of cohort observation studies assessing the effect of milk on various-cause mortality

With regards to prospective cohort studies assessing the effect of milk and dairy products consumption on various causes of mortality in adults, and despite some conflicting observations, meta-analysis tends to agree that regular milk intake

2095 especially low-fat incorporated in a healthy diet may be beneficial in lowering the
2096 risk of cardiometabolic disease, improving frailty indices in the general adult
2097 population and potentially contributing to cognitive function in elderly (Camfield *et*
2098 *al*., 2011; Visioli and Strata, 2014).

2099 Authors have suggested, as explanation for conflicting results relating to the effect of
2100 milk and dairy products on human health, the adjustments for various confounding
2101 factors in prospective cohort studies and the consecutive adjustments in meta-
2102 analysis. Background diet, socioeconomic status, smoking and life-style, baseline
2103 measurements of health status, differences in self-reported portion measurement and
2104 lack of discrimination between whole or low-fat dairy product are some of the
2105 possible confounding factors reported in the prospective studies and meta-analysis
2106 studies considered in this review that may have led to conflicting results or masking
2107 potential effects of dairy consumption. Interestingly, some studies report that milk
2108 and dairy products consumers had higher education and socioeconomic status as well
2109 as enjoying a healthier life-style compared to those with lower frequency dairy
2110 product consumption (Wu *et al*., 2016), whereas in other studies milk consumption
2111 was higher among people of lower socioeconomic status (Talaei *et al*., 2017).

2112 Milk contains a wealth of nutrients that may synergistically and/or individually
2113 mediate the reported potential effects of milk and dairy products ingestion in human
2114 health. Various peptides such as lactoferrin, α-lactalbumin, β-lactoglobulin, fractions
2115 of α-, β- and κ-casein and peptides released from dairy after digestion, gut
2116 microbiota fermentation and food preparation, have been reported to play a
2117 beneficial role as anti-thrombotic compounds, inhibiting angiotension and potentially
2118 as insulintropics (reviewed in Horner *et al*., 2016). Calcium, vitamin D and
2119 conjugated linoleic acid (CLA) may be mediators of the chemoprotective activity of

milk (Song *et al*., 2015). Conversely, D-galactose, lactose, IGF-1, calcium, saturated fat and hormones contained in milk have been proposed as factors with detrimental effects in oxidative stress and cancer incidence (Yang *et al*., 2016(a); Yang *et al*., 2016(b); Berghlodt *et al*., 2017). Well-designed prospective studies adjusting for a wide range of confounding factors and clinical trials on selected cohorts and *in vitro* /*vivo* studies will further elucidate the role of whole dairy and individual milk components in human health and reveal the mechanism that mediate the effects.

1.3.1.5 Lactose-intolerance

Dietary oligosaccharides and disaccharides are hydrolysed by intestinal disaccharidases in order for the constituent monomers to be absorbed by the enterocytes in the small intestine (Amiri *et al*., 2015). Oligosaccharides and disaccharides that escape hydrolysis and absorption in the small intestine reach the distal colon where they can cause osmotic flux of water and increased H_2, CO_2 and fatty acids concentration due to fermentation by colonic bacteria (Amiri *et al*., 2015). The disaccharide lactose (consisting of galactose and glucose) is the main carbohydrate in human and farm animal milk (Silanikove *et al*., 2015). In humans, hydrolysis of lactose is highly active during lactation and declines after weaning; more than half of the human population has low lactase activity as a result of the genetically programmed decline of lactase synthesis (lactose non-persistent subjects - LNP) (Deng *et al*., 2015). Lactase deficiency may also occur after a GIT disease or as a rare congenital condition in early life; in all cases malabsorbed lactose persists in the distal GIT and in some cases it may lead to symptoms of lactose intolerance (Deng *et al*., 2015). Not all adults lose lactase synthesis activity and the ability to thoroughly digest lactose (lactose persistent subjects- LP). In modern populations

deriving from ancient pastoral populations, lactase synthesis may remain active throughout adulthood (Gerbault *et al*., 2011).

Lactose malabsorption in LNP individuals does not always lead to lactose intolerance symptoms and to a clinically detectable condition (Misselwitz *et al*., 2013). Lactose maldigestion can be accompanied by bloating, abdominal pain, borborygmi, diarrhoea and nausea, and subjects reporting such discomforts may follow certain diagnostic steps towards the clinical evaluation of their condition (summarized by Misselwitz *et al*., 2013). The diagnosis includes tests for malabsorption such as the lactose H_2-breath test alone or after the ingestion of milk. Throughout the lactose intolerance diagnosis procedure, other conditions such as IBS or small intestinal bacterial overgrowth (SIBO) should be considered as potential contributors to the self-reported discomforts (Misselwitz *et al*., 2013).

People with self-reported symptoms of lactose malabsorbance or lactose intolerance tend to avoid milk and dairy products therefore excluding from their daily diet a valuable source of important nutrients and calcium (Heaney, 2013). Avoidance of dairy foods is not recommended for the improvement of lactose intolerance (Szilagyi, 2015a) especially when, apart from lactose malabsorption or intolerance, there is another condition that could be improved by dairy consumption (Szilagyi *et al*., 2016). Lactase non-persistent individuals can tolerate an average of 12 g of lactose per day (approximately one glass of milk) without developing symptoms (Szilagyi, 2015b). Lactase non-persistent individuals can develop tolerance to lactose through adaptation of the colonic microbiota to lactose fermentation, a phenomenon that indicates that lactose may have a prebiotic effect on the gut microbiota of LNP subjects (Szilagyi, 2015b).

1.3.1.6 Milk and the gut microbiota

Little is known about the effect of non-human fluid non-fermented milk on the human gut microbiota and the prebiotic potential of its carbohydrate load. The carbohydrate fraction of milk consists mainly of lactose, oligosaccharides (OS) and glycoconjugates (**Table 4**). Mature human milk has higher content of lactose (70 g/l) and OS (5-15 g/l) compared to bovine milk (48 g/l and 0.05 g/l respectively) (summarized in Bode, 2012). Glucose, galactose, fucose, *N*-acetylneyraminic acid (NeuAc/sialic acid) and *N*-acetylglucosamine (GlcNAc) are the simple carbohydrates that compose the milk OS; *N*-glycolylneuraminic acid (NeuGc) and lactose amine are present only in bovine milk (Tao *et al*., 2008). The human milk oligosaccharides (HMOs) are mostly fucosylated whereas a high proportion of the bovine milk oligosaccharides (BMOs) is sialylated (Bode, 2012).

Fructo-oligosaccharides and GOS that are widely used as prebiotics (see Chapter 1.2) do not reach the structural and compositional complexity of HMOs or the (similar in structure) highly complex BMOs (Zivkovic and Barile, 2011). Human milk oligosaccharides have been extensively studied because of their potent prebiotic effect on the infant gut microbiota (Smilowitz *et al*., 2014). If BMO prebiotic activity is proven, BMOs could then be used as an alternative to HMOs for food supplementation and the dairy industry could scale-up the relevant BMO isolation (Zivkovic and Barile, 2011).

Bovine milk OS exist only in trace amounts in mature bovine milk, and whey concentrate that naturally contains large amounts of OS (approximately 200 mg/L) is currently used for BMO isolation (Zivkovic and Barile, 2011). There are ongoing efforts to develop scalable methods to isolate highly pure BMOs and sensitive

analytical tools for the characterisation of the OS isolate profiles in order to further elucidate the structural complexity and natural variation extend of BMOs (Sundekilde *et al*., 2012; Mehra *et al*., 2014; de Moura Bell *et al*., 2018). Importantly, only a few BMOs (approximately 40) have been identified compared to the more than 100 known HMOs (Bode, 2012).

To my knowledge there are no extensive studies yet exploring the effect of non-human non-fermented fluid milk on the gut microbiota. Tannock *et al*. (2012) reported that the faecal microbiota of bovine milk supplemented formula-fed infants had relatively greater abundance of *Lachnospiraceae* compared to breast-fed. Yin *et al*. (2014) reported that ingestion of acidified milk-supplemented standard chow increased the abundance of *Lachnospiraceae* in the murine faecal microbiota compared to milk supplemented with probiotic *Lactobacillus* or water.

A few recent studies exploring the effect of isolated BMOs on the gut microbiota gave some insight on the beneficial potential of milk carbohydrates in GIT health. Charbonneau *et al*. (2016) showed that in both germ-free mice and gnotobiotic piglets, sialylated BMO (S-BMO) supplemented diet resulted in a faecal microbiota-mediated attenuation of the effects of malnutrition on lean body mass, muscle and bone health and cognitive function. Karav *et al*. (2016) demonstrated *in vitro* that OS derived from bovine milk glycoproteins supported *Bifidobacterium longum* subsp. *infantis* growth. *Bifidobacterium infantis* is a member of the gut microbiota of breast-fed infants and selectively grows on HMOs. Bovine milk glycans may represent a selective substrate for gut microbiota taxa alternative to HMOs (Karav *et al*., 2016).

Boudry *et al*. (2017) reported that BMOs normalized diet-induced obesity effects on the gut microbiota composition and gut barrier function in a murine model. Similarly, Hamilton *et al*. (2017) showed that, in a mouse model, BMO supplemented diet was more efficient in attenuating the effect of high fat diet on gut barrier function and gut microbiota compared to prebiotic inulin supplementation. A recent study investigating the effect of dietary supplementation with BMOs in humans showed that equivalent to plant-origin prebiotic doses of BMOs were well tolerated by healthy adults but with no drastic effect on stool consistency and gut microbiota composition after 11 days of dietary intervention (Smilowitz *et al*., 2017).

Other components of milk may confer gut microbiota-mediated health benefits. Norris *et al*. (2016) reported that a bovine milk sphingomyelin-supplemented diet counteracted the negative effect of high-fat diet in a mouse model by lowering the serum cholesterol and by reducing the circulating LPS with a concomitant increase in the relative abundance of faecal Firmicutes and Actinobacteria, predominantly *Bifidobacterium*. In a neonatal murine model, bovine MFGM-supplemented formula effectively attenuated the GIT deformities observed under the influence of MFGM-deprived diet; the gut microbiota composition of the mice fed the MFGM supplemented formula clustered with the faecal microbiota of mice fed maternal milk that contains naturally occurring MFGM (Bhinder *et al*., 2017). Lee *et al*. (2018) observed improvement of metabolism and changes in microbial metabolites in malnourished children due to bovine MFGM supplementation. Conversely, in a murine model, ingestion of a diet high in saturated milk fat resulted in the increased abundance of the sulphur-reducing bacterium *Bilophila wadsworthia* in the faecal microbiota, and increased the incidence of colitis in genetically susceptible mice but not in wild type (Devkota *et al*., 2012).

Exploring the effect of individual milk components on the gut microbiota and health is useful on its own merit but it does not allow for a deep comprehension of the synergistic effect of milk components. Despite some research on the effect of milk components on the GIT and the gut microbiota, the synergistic effect of all the constituent components of fluid milk and variations of the standard bovine milk such as whole and lactose free, remains largely unexplored.

1.3.1.7 The prebiotic potential of the dairy product Glycomacropeptide (GMP)

Glycomacropeptide (GMP) is the glycosylated form of the caseinomacropeptide (CMP) that is released in whey during cheese production when chymosin (rennin) hydrolyses milk kappa casein (κ-CN) into para-κ-CN and caseinomacropeptide (CMP) fractions (Neelima, 2013). The genetic variations of the amino acid (AA) sequence of κ-CN and the variant phosphorylation and glycosylation sites contribute to the heterogeneity of the GMP peptides (reviewed in Thoma-Worringer *et al*., 2006). Approximately 50% of the bovine CMP is glycosylated with 5 variations of *O*-linked mono- to tetra-saccharide carbohydrate chains containing combinations of NeuAc, Gal and GalNAc (Thoma-Worringer *et al*., 2006). Glycomacropeptide has a high content of essential and branched chain AA (EAA and BCAA respectively) compared to the average dietary protein and depending on the efficiency of GMP isolation and purification, none to trace amounts of the aromatic AA phenylalanine (Phe), tyrosine (Tyr) and tryptophan (Trp) (Etzel, 2004). In whey, GMP makes up approximately 25% of the protein content, and ultrafiltration of whey is recommended as the most efficient method for the commercial recovery of GMP (Neelima, 2013).

Glycomacropeptide has a number of health relevant properties. Based on a number of *in vitro* studies it was suggested that GMP may inhibit binding by *Cholera* toxin, *Shigella flexneri*, *Salmonella enteritidis* and pathogenic *E. coli* to the mammalian cell mostly due to its sialic acid content (Kawasaki *et al*., 1992; Oh *et al*., 2000; Nakajima *et al*., 2005; Bruck *et al*., 2006; Feeney *et al*., 2017). Glycomacropeptide exerts some effect on the immune system as demonstrated in *in vitro* studies (Requena *et al*., 2009; Gong *et al*., 2014). In murine models of induced colitis and ileitis, GMP administration normalized the systemic and local inflammation and attenuated the macroscopically observed GIT damage due to the inflammatory treatment (Requena *et al*., 2008; Lopez-Posadas *et al*., 2010; Xu *et al*., 2013; Ortega-Gonzalez *et al*., 2014; Cui *et al*., 2017). Hvas *et al*. (2016) observed the anti-inflammatory potential of GMP administration on patients with UC. Glycomacropeptide has been successfully used in clinical trials as a naturally occurring, low in Phe, dietary alternative to synthetic AA protein for phenylketonuria (PKU) dietary management (Ney *et al*., 2016; Ahring *et al*., 2017).

There is inconclusive evidence on the prebiotic potential of GMP. Supplementation of growth medium with GMP could sustain the growth of probiotic strains of *Lactobacillus* and *Bifidobacterium* in pure culture conditions (Robitaille, 2013). The growth promoting effect of GMP on probiotic *Lactobacillus* and *Bifidobacterium* taxa was not confirmed when complex faecal microbiota from either infants or adults was used for *in vitro* fermentations of GMP supplemented media (Bruck *et al*., 2006; Hernandez-Hernandez *et al*., 2011; Ntemiri *et al*., 2017; Chapter 2, this thesis).

In a piglet model GMP feeding resulted in the increase of *Lactobacillus* counts and decrease of pathogenic *E.coli* (Hermes *et al*. 2013). Sawin *et al*. (2015) reported that

wild type (WT) and PKU mice fed GMP-supplemented diet had significantly reduced relative abundance of the sulphur-reducing taxon *Desulfovibrio* in the faecal and caecal microbiota compared to mice fed casein and AA supplemented diet respectively. In the caecum and faecal microbiota of PKU mice fed GMP-supplemented diet the relative abundance of Bacteroidetes was significantly higher compared to an AA-based feeding regime; increase in Bacteroidetes was not observed for WT mice fed GMP (Sawin *et al*., 2015). Sawin *et al*. (2015) provided valuable insight on the effect of GMP on the murine faecal and caecal microbiota in comparison to the AA and casein effect. However, the group did not provide compositional information of the baseline microbiota of WT and PKU and did not present microbiota data at a fine taxonomical level which would have put in context the observed effect of the diets on the microbiota. Recently, Jimenez *et al*. (2017) using an allergy rat model observed that GMP administration conferred significant increase in the faecal *Lactobacillus* and *Bifidobacterium* population. *Bacteroides* population also increased in rats with allergy after GMP administration (Jimenez *et al*., 2017). These two recent papers report an effect of GMP on the Bacteroidetes population; baseline microbiota composition information is necessary in order to clarify what compositional "dysbiosis", if any, was restored with GMP administration.

1.4 Aims and objectives

In this chapter have reviewed the general aspects of the gut microbiota composition, how the microbiota is established in the human gut at birth and during the first years of life, and how the gut microbiota composition and functionality is associated with health and disease throughout life span, and with ageing. Importantly, changes in the gut microbiota composition and functionality are associated with age-related frailty. The gut microbiota is a modifiable factor for maintaining health and ameliorating disease, and one of the means to modify the microbiota is the use of dietary components with prebiotic potential. This research thesis focused on exploring the potential of certain dietary substrates of animal- and plant-origin to modulate the gut microbiota of older people aiming at identifying changes in the gut microbiota composition that can be associated to a "healthy" gut microbiota profile. The dietary substrates of interest were the milk-derived glycomacropeptide (GMP), whole milk (with or without lactose) and blueberry fruit. Milk is a source of nutrients including carbohydrates and oligosaccharides but the prebiotic potential of milk and its by-products remains largely unexplored. Blueberries are rich in polyphenols; these compounds can modulate the gut microbiota but there are no clear associations between microbiota members and the bioconversion of phenolic compounds. Importantly, the potential of "dysbiotic" faecal microbiota to respond to dietary supplementation was investigated. An *in vitro* colon model was established in order to evaluate the prebiotic potential of the dietary substrates before proceeding to an *in vivo* model.

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Chapter 2

Glycomacropeptide sustains microbiota diversity and promotes specific taxa in an artificial colon model of elderly gut microbiota

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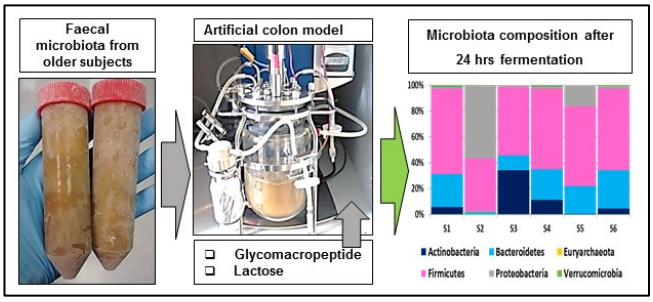


2.1 Abstract

The potential of milk-derived glycomacropeptide (GMP) and lactose for modulating the human gut microbiota of older people, in whom loss of diversity correlates with inferior health, was investigated. We used an *in vitro* batch fermentation (artificial colon model) to simulate colonic fermentation processes of two GMP products, i.e. a commercially available GMP concentrate and a semi-purified GMP concentrate, and lactose. Faecal samples were collected from healthy and frail older people. Samples were analysed by Illumina Miseq sequencing of rRNA gene amplicons. The commercial GMP preparation had a positive effect on the growth of *Coprococcus* and *Clostridium* cluster XIVb and sustained a higher faecal microbiota diversity compared to control substrates or lactose. Lactose fermentation promoted the growth of Proteobacteria including *Escherichia/Shigella.* This work provides an in-depth insight on the potential of GMP and lactose for modulating the gut microbiota and contributes more evidence confirming the prebiotic activity of GMP.

Keywords: Glycomacropeptide, lactose, artificial colon model, faecal microbiota, healthy elderly, frail elderly

2.2 Introduction

The human gastrointestinal tract (GIT) harbours a phylogenetically diverse bacterial community that significantly contributes to host health (Donaldson *et al*., 2015).The gut microbiota is determinatively shaped by habitual diet (Claesson *et al*., 2012; Jeffery *et al*., 2016; Sonnenburg *et al*., 2016). Dysbiosis refers to alterations from typical proportions of taxa in the gut microbiota composition and altered diversity, changes often related to non-healthy conditions (Cho *et al*., 2012). Targeted modulation of the gut microbiota is emerging as an approach for ameliorating a series of conditions related to dysbiosis (Foxx-Orenstein *et al*., 2012).

There is evidence linking the process of ageing-related health loss to the gut microbiome (Heintz *et al*., 2014). From adulthood to older age the gut microbiota undergoes significant compositional and metabolic changes that define the gut microbiota profile of the elderly (Claesson *et al*., 2011; O'Tole and Jeffery, 2015). These shifts in the gut microbiome increase pathobiont infection susceptibility in the older population (Rea *et al*., 2012).

Recent studies suggest a link between dairy consumption and a number of health benefits including muscle maintenance and bone density in older subjects (Sahni *et al*., 2014; Lana *et al*., 2015). Little is known about the effect of milk components on the gut microbiota. The content of lactose in commercial milk is ~4% (Albenzio *et al*., 2016). Lactose is a candidate prebiotic (Roberfroid, 2007) especially among lactase non-persistent people (LNP) related to its demonstrated growth promotion of lactobacilli and bifidobacteria (Szilagyie *et al*., 2015), with a potential protective role in colorectal health (Jatvinen *et al*., 2001).

Caseinomacropeptide (CMP) is produced from milk during cheese making; during this procedure, chymosin hydrolyses the kappa-casein (κ-CN) of milk resulting in the para-κ-CN (residues 1-105) and CMP (residues 106-169) (Neelima *et al*., 2013). Glycomacropeptide (GMP) is the glycosylated form of CMP; GMP has five mucin-type glycans namely *N*-acetylneuraminic acid (NeuAc) or sialic acid, galactose (Gal), and N-acetylgalactosamine (Gal-Nac) that anchor to the CMP through *O*-glycosidic linkages (Kreuss *et al*., 2008). GMP is rich in branched chain amino acids (BCAA) and essential amino acids (EAA) and purified GMP concentrate is depleted of aromatic amino acids (Thoma-Worringer *et al*., 2006).

The major health-relevant effects of GMP are anti-inflammatory activity, protection against GIT pathogens and contribution to phenylketonouria (PKU) nutritional management (Thoma-Worringer *et al*., 20106; Van Calcar and Ney, 2012). There are contradictory data on the effects of GMP on the growth promotion of *Bifidobacterium* and *Lactobacillus* strains (Bruck *et al*., 2006; Hernandez-Hernandez *et al*., 2011; Robitaille, 2013). Recently Sawin *et al*. (2015) reported that GMP feeding resulted in a significant decrease in the abundance of Proteobacteria and the genus *Desulfovibrio* in the caecal and faecal microbiota of wild type and phenylketonouric mice. Although the data were based on murine microbiota and no extended description of the microbiota was provided, the study offered a more conclusive view of the effect of GMP on the gut microbiota compared to previous studies.

The aim of this study was to explore the prebiotic potential of two GMP products, a commercially available product and a semi-purified preparation, and of lactose, on the faecal microbiota in an *in vitro* batch fermentation system over 24 hrs. The

responsiveness to these growth substrates of low diversity and higher diversity microbiota was investigated in faecal samples obtained from healthy and frail elderly subjects, since the latter are characterised by reduced microbiota diversity.

2.3 Material and Methods

Faecal samples: Faecal samples were collected from healthy older donors (community samples) (EM425 male 81 yrs, EM278 female 69 yrs, EM604 female 75 yrs) and older donors that reside in long term residential care units (longstay samples) (EM703 male 93 yrs, EM297 female 82 yrs, EM704 male 89 yrs). All practices were approved by the local Clinical Research Ethics Committee. Each stool sample was placed in a container with a reducing agent (Anaerocult A; Merck Millipore, Ireland) and transferred to an anaerobic cabinet less than an hour after defecation. Each stool was thoroughly homogenised (10% w/v) in sterile reduced phosphate buffer saline (PBS) and 20% glycerol. Faecal slurries from each stool sample were prepared as inocula for the fermentation vessel and stored frozen in aliquots at -80 °C before thawing and adding to the fermenter.

Commercial and semi-purified GMP: For the *in vitro* tests, Lacprodan CGMP-10 (CGMP-10) was obtained from Arla Foods Ingredients, Viby J, Denmark. The semi-purified GMP (tGMP) was produced as described below. Sweet whey protein isolate powder (sWPI) was sourced from Kerry Group plc, Listowel Ireland. A GMP enriched whey fraction was prepared using a modified method as developed by Tanimoto *et al* (1991). Three hundred grams of sWPI was reconstituted in 1 L distilled water, stirred at room temperature for 2 hrs and left to rehydrate at 4 °C overnight. The sWPI was pH adjusted to 3.8 using 1 M HCl and stirred at room temp for 30 min. The sWPI was then processed through a 30 kDa molecular weight cut off

(MWCO) membrane (Vivaflow 200 tangential flow units; Sartorius, Göttingen, Germany) at an operating TMP of ~2.25 bar, and 300 mL of permeate (Pr1) was collected. Peristaltic pumps (230V Masterflex L/S Easy-Load peristaltic pumps; Cole Parmer, Niles, IL, USA) and size 16 PVC pump tubing was used. Pr1 was then pH adjusted to pH 7.0 using NaOH and processed using 10 kDa MWCO polyethersulfone membrane following 30 min acclimatisation period in full recirculation mode, and the GMP enriched retentate was collected. Diafiltration was carried out with equivalent volume of distilled water and diluted retentate was concentrated back to its original volume and frozen at -20°C. GMP enriched solutions were freeze dried using Wizard 2.0 freeze drier (Virtis AdVantage, UK). Quantification of the whey proteins α-lactalbumin, β-lactoglobulin and GMP (for both GMP products) was carried out by RP-HPLC using automated Waters 2695 Separation module (Waters Associates, Milford, MA, USA) fitted with a quaternary pump, mobile phase degasser, auto-sampler and a multi-wavelength detector. Calibration curves were obtained for each of the standards i.e. α-lac, β-lg and GMP and Waters Empower data analysis software was used to quantify the α-lac, β-lg and GMP content.

In vitro **fermentations:** A single stage chemostat system and basal medium (no carbohydrates supplementation) was used as described previously to simulate the colonic microbiota fermentation of the selected substrates (Hernandez-Hernandez *et al.*, 2011). The pH (pH 7.0) and temperature (37°C) controlled batch fermentations were conducted in 1 L working volume, under continuous flow of CO_2 over a period of 24 h. Twenty-four hr fermentation was chosen over a continuous fermentation as a standard approach to study prebiotic effects on the faecal microbiota (Hernandez-Hernandez *et al.*, 2011; Zhang *et al.*, 2016). [22, 26] Cysteine-HCL was filter sterilised

(0.2 μm filters) and added to the basal medium after sterilisation. Glucose and lactose were filtered sterilised and added to the sterilised medium at a final concentration of 1% w/v. GMP preparations were added to the sterilised medium after dilution in sterile distilled water. Six substrate conditions were used for each of the faecal samples: i. basal medium, ii. Glucose (Sigma-Aldrich), iii. lactose (Sigma-Aldrich), iv. CGMP-10, v. tGMP, vi. CGMP-10 combined with glucose. All substrates were added to a final concentration of 1%; for substrate condition vi. CGMP-10 and glucose were added to 0.5% respectively. The faecal samples were thawed in an anaerobic cabinet and inoculation in the fermenter vessel was performed anaerobically immediately after thawing at a final concentration of 1% w/v. At time points zero hours and twenty-four hours 50 mL of fully stirred fermentation culture was retrieved and immediately centrifuged. Faecal pellet and supernatant were kept separately in -20 °C for genomic DNA (gDNA) extraction and SCFA analysis respectively.

Genomic DNA extraction: Genomic DNA (gDNA) was extracted using the QIamp Fast DNA Stool (Qiagen) kit. Faecal pellets were weighed and suspended in 1 ml sterile PBS. 200 μL of the suspended slurry was retrieved for gDNA extractions in order to provide the 200 mg of faeces required for the QIamp Fast DNA Stool (Qiagen) extraction kit protocol. The faecal slurry was placed in sterile tubes containing 0.1 mm, 0.5 mm and 1.0 mm zirconia / glass beads (Thistle Scientific, UK). One ml of InhibitEX buffer was added to the samples and then homogenised under two pulses of 1 min and a final of 30 sec using a Minibeadbeader (Biospec Products). In the intervals of the homogenisation steps the samples were placed on ice for 1 min. The samples were then placed in a 70 °C heat-block for 10 min. The following steps of the DNA extraction were carried out as described in the Qiagen

protocol using 15 µL of proteinase K with 200 µL of AL buffer, 200 µL of lysate and 200 µL of ethanol at the relevant steps.

16S rRNA amplicon Illumina library preparation: The Illumina MiSeq System (San Diego, California, USA) was used for amplicon sequencing of the 16S rRNA variable region V3 and V4. The universal 16S ribosomal RNA gene primers forward primer for V3 region 5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCC TACGGGNGGCWGCAG 3' and reverse primer for V4 region 5' GTCTC GTGG GCTC GGAG ATGTGTATA AGA GACAGGACTAC HV GGG TATC TA ATCC 3' were used (Klindworth *et al*., 2013). The library preparation and sequencing was performed according to the Illumina MiSeq System protocol. The PCR products were purified and dual-index barcodes attached to the amplicon (Nextera XT V.2 Index Kits sets A and D, Illumina). Amplicons were purified using Agencourt AMPure XP-PCR Purification system (Beckman Coutler, Inc.) and quantified using a Qubit dsDNA HS Assay Kit (Thermo Fischer Scientific, MA, USA). Samples were pooled in order to achieve the same concentration of DNA per amplicon and sequenced by Eurofins Genetics Services Ltd. (Wolverhampton UK) on a MiSeq Illumina platform and 2x300 bp Chemistry.

16S amplicon sequence analysis: For all demultiplexed samples barcodes were reattached with relevant custom scripts (Flemer *et al*., 2016), primers were removed by the cutadapt tool, forward and reverse reads per sample were joined using the FLASH computational tool (Magoc *et al*., 2011). The reads were demultiplexed using the split_libraries.py script in Qiime. *De novo* operation taxonomic unit (OTU) table based on the data reads was generated via Usearch; reads were assigned to OTUs as follows. Based on length size unique sequences were selected using the

derep_fulllength command discarding singletons by –minuniquesize -2 command. Clustering of the sequences was performed using cluster_otus command (Edgar *et al*., 2013). Sequences were screened for chimeras using the ChimeraSlayer utility (Haas *et al*., 2011). To map all reads to the representative sequences OTUs database the Usearch_global command was used based on 97% identity threshold. The uc file generated was converted to an OTU table via uc2otutab.py command. Taxonomy was assigned to each OTU (classify.seqs) to genus level using Mothur and the RDP 16S rRNA database (Wang *et al*., 2007).

Microbiota composition statistics: The R software package was used for the statistical analysis of the sequenced data (R Core Team, 2014). From the sequenced samples only those with a number of reads >5000 were kept for further analysis. The OTU counts per sample were rarefied to a common minimum of reads per sample. Metrics of phylogenetic distance between the sequenced samples were calculated using the weighted and unweighted UniFrac method (Lozupone and Knight, 2008). The data was visualised using principal components analysis (PCoA) with functions s.class and dudi.pco from the ade4 package. The row counts data table was transformed to relative abundances (0:100) table for obtaining relative abundance of the bacterial populations in the samples to genus level. Relative abundances results were obtained for individual samples and aggregated community-type and longstay-type samples. The Shannon diversity index was used as a metric of within-sample alpha diversity and species evenness (Lozupone and Knight, 2008). A pairwise Wilcoxon rank test was performed to identify significant Shannon diversity changes from 0 hrs to 24 hrs due to medium fermentation. The DESeq2 package (Love *et al*., 2014) was used for pairwise comparisons of 0hrs and 24hrs for the fold changes (log2 fold change) in the bacterial populations at genus level based on row read

counts per sample. Normalisation of read counts is embedded in the DESeq2 function. P value for DESeq2 results was adjusted for multiple comparisons using the Benjamini-Hochberg method. Results were considered significant if the p adjusted values were padj≤ 0.05 while padj <0.1 were regarded as a trend.

Short chain fatty acid analysis and statistics: High performance liquid chromatography (HPLC) was used for the analysis of acetate, propionate and butyrate production (Hernandez-Hernandez *et al*., 2011). Slurry retrieved from the fermenter vessel was centrifuged for 30 min. The supernatant was filtered through 0.2 μm filter and 1 ml was used for SCFA analysis HPLC. An agilent 1200 HPLC system with a refractive index detector was used. An Agilent Hi-Plex H 300x7.7mm column was used with 0.01N H_2SO_4 elution fluid, at a flow rate of 0.6 mL/min and column temperature at 65°C. Graph Pad Prism 5 Software was used for the analysis and visualisation of the HPLC results. A Kruskal-Wallis non-parametric test with a post hoc Dunn's test was applied using GraphPad Prism 5 in order to compare the SCFA production in the faecal microbiota after 24 hrs fermentation with the supplements against glucose-supplemented medium fermentation.

2.4 Results

Chemical composition of GMP preparations

Two GMP preparations were investigated in this study, a commercial fraction obtained from Arla Foods Ingredients called Lacprodan CGMP-10 (CGMP-10), and a GMP preparation prepared in our laboratories in Teagasc Moorepark Food Research Centre (tGMP). The chemical composition of these two GMP products is shown in **Table 5**. CGMP-10 had a higher content of GMP (70.2%) and lower content of lactose (<2%) compared to tGMP with 51.4% and 4.4% respectively. The

protein content in CGMP-10 and tGMP was 80-84% and 65.6% respectively. The two products varied in their content of the whey proteins beta-lactoglobulin (β-lactoglobulin) and alpha-lactalbumin (α-lac) with 10.6% and 8% respectively for CGMP-10 and 1.5% and 19.3% respectively for tGMP. To investigate the feasibility of using less purified GMP, both preparations were carried forward into the artificial colon model.

Table 5 Chemical composition of the two glycomacropeptide products used in this study, Lacprodan CGMP-10 and semi-purified tGMP.

Component	*CGMP-10*	*tGMP*
β-lg[a]	10.63%	1.48%
α-lac[b]	8.02%	19.32%
GMP	70.22(±4.7)%	51.39(±7.1)%
Lactose	<2%	4.4%
Total protein	80-84%	65.61%

[a]***β-Ig****, beta lactoglobulin.* [b]***α-Lac****, alpha lactalbumin.*

Compositional differences between the faecal microbiota from healthy and frail older donors

We have previously reported that elderly subjects living in long-term residential care units have a lower diversity microbiota of a characteristic composition compared to that of community dwelling subjects (Claesson *et al*., 2012; Jeffery et al., 2016).The compositional differences include significantly reduced Lachnospiraceae abundance in the microbiota of longstay subjects compared to community including genera such as *Coprococcus* and *Roseburia*, and significant enrichment in Proteobacteria abundance including Enterobacteriaceae. We re-enrolled 3 community-dwelling and 3 long-term care unit residing ELDERMET (Claesson *et al*., 2012) subjects (i.e., a

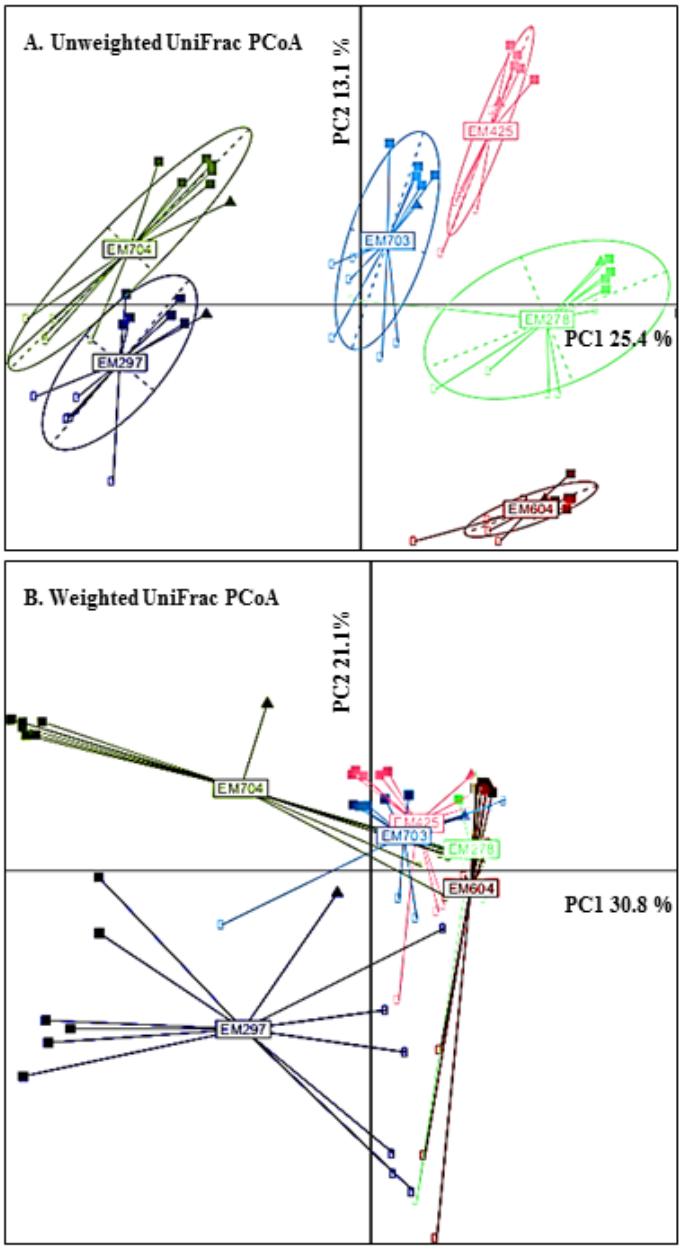
cohort of 500 elderly whose gut microbiota composition was characterised and link between health and microbiota was investigated) and re-examined their microbiota several years after initial analysis to ensure that thecomposition had the expected profile as described in previous studies (Claesson *et al*., 2012; Jeffery *et al*., 2016).

Based on the principal coordinates analysis (PCoA) of the Weighted (**Figure 3 A**) and Unweighted UniFrac (**Figure 3 B**) distances based on the sequenced 16S rRNA gene sequences in the faecal samples at baseline (time point 0 hrs), two of the samples obtained from frail elderly (EM297 and EM704) clustered closer together and separated from the other four samples (EM703, EM425, EM604, EM278). Thus, one of the longstay subjects had a community type microbiota. For the downstream analysis of the samples collected after 24 hrs of *in vitro* fermentation, the samples were aggregated as follows. Samples EM297 and EM704 were aggregated into longstay type (LS), and sample EM703 obtained from a frail individual was considered together with EM425, EM604, EM278 obtained from healthy older individuals as community type (COM). Across all six medium / supplementation combinations, all six time point 0 hrs microbiota clustered by donor, indicating an accurate baseline microbiota / inoculum for each fermenter condition tested.

The composition of the community and longstay type faecal microbiota at phylum and family level is shown in **Figure 4** and **5** respectively. The two types of microbiota had distinct differences. Longstay type samples were significantly enriched for Euryarchaeota (average 27% relative abundance) and Proteobacteria (average 14% relative abundance) compared to the community type microbiota. The community type microbiota had higher relative abundance of Firmicutes (approximately 68%) and Bacteroidetes (approximately 22.5%) compared to an

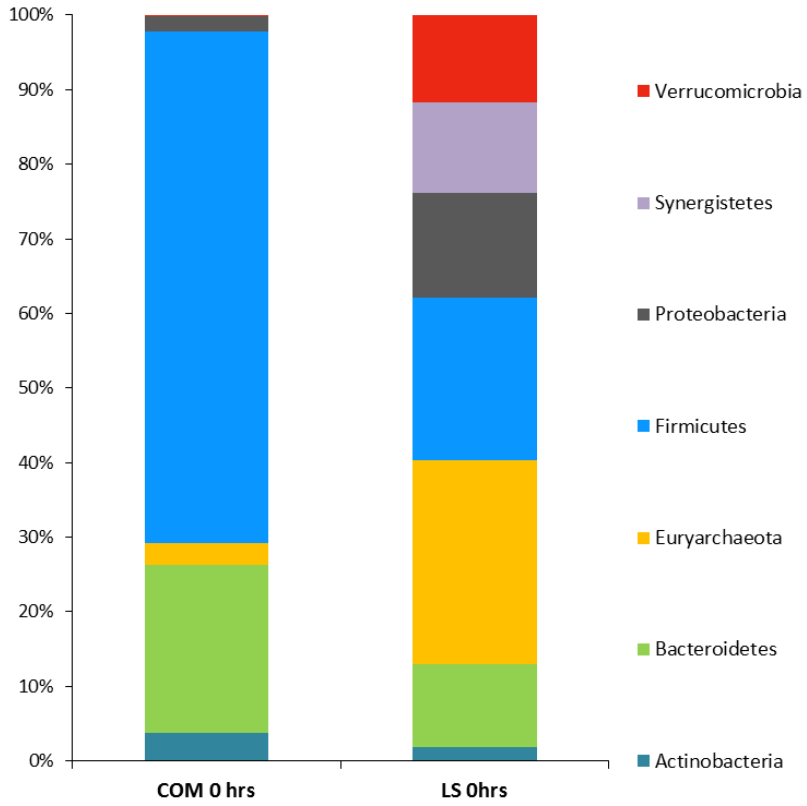
average 22% and 11% respectively in the longstay type microbiota. At family level Lachnospiraceae comprised up to around 37% of the community type microbiota whereas in longstay type microbiota, Lachnospiraceae were present with an average 4% relative abundance. Other distinct differences between the two sample types were the relative abundance of the Ruminococcaceae family, average 14% in community type and 6% in longstay type, and the enrichment in the longstay type faecal microbiota of the Verrucomicrobiaceae, Synergistetes and Methanobacteriaceae compared to community type (**Figure 5 B**).

The observations here are broadly consistent with our previous description of microbiota composition in frail elderly subjects, allowing us to conclude that the available samples were suitable representatives to allow for the investigation of the effect of the selected dairy ingredients on the microbiota of elderly people.



3421

3422 **Figure 3 Principal coordinates analysis of UniFrac Unweighted (A) and**
3423 **Weighted (B) distances.** "■" time points 0 hrs and "□" time points 24 hrs, "▲"
3424 stool microbiota before faecal slurry preparation.



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3426 **Figure 4 Composition of the baseline (0 hrs) faecal microbiota at Phylum level.**
3427 The relative abundance of the various phyla is shown for aggregated community
3428 (COM; EM425, EM278, EM604 and EM703) and aggregated longstay (LS; EM297,
3429 EM704) faecal microbiota. Only phyla with relative abundance ≥0.5% are shown.

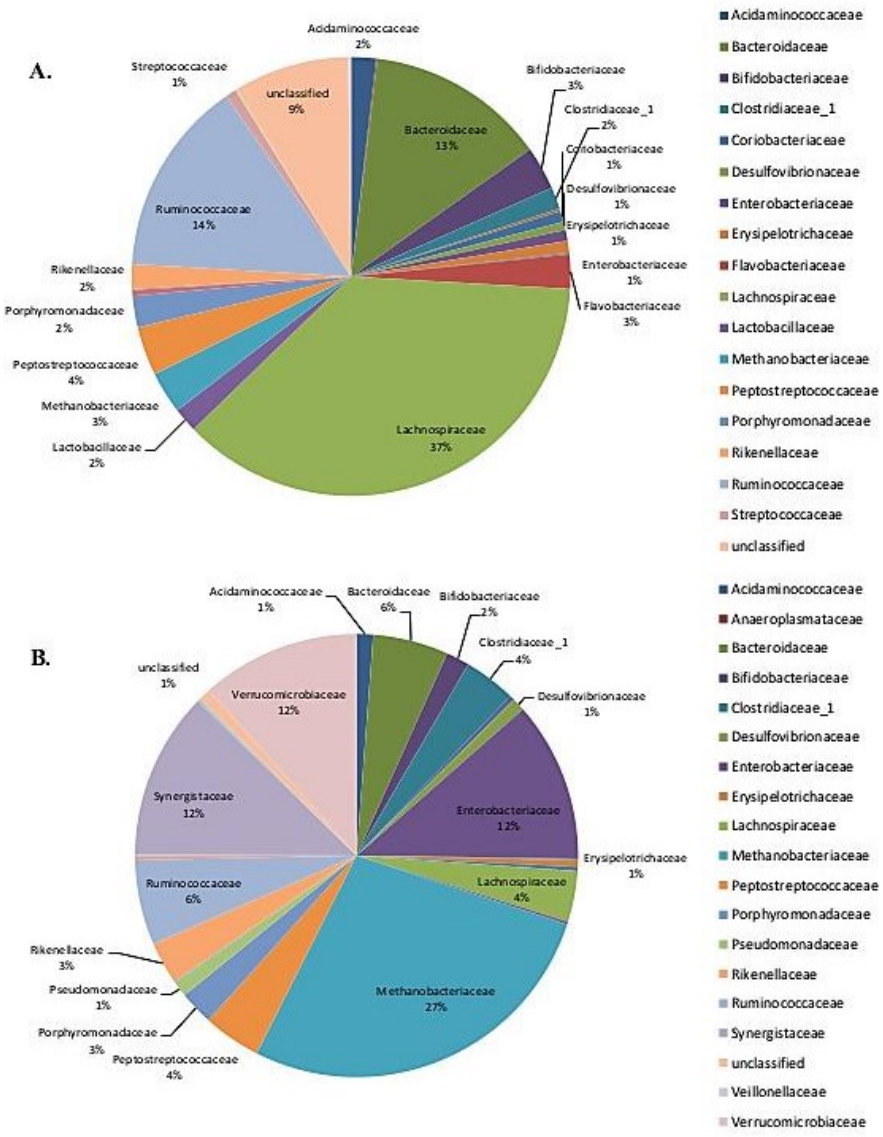


Figure 5 Composition of the baseline (0hrs) faecal microbiota at Family level. The relative abundance of the various families is shown for (A) aggregated community (EM425, EM278, EM604 and EM703) and (B) aggregated longstay type faecal microbiota (EM297, EM704).

GMP supplementation sustained a higher diversity faecal microbiota compared to other media

We tested the development of the microbiota in the artificial colon in a basal medium without carbohydrates supplementation, compared to five supplementation regimes

with the two GMP preparations, glucose (a common carbohydrate of typical human diet) and lactose. Regardless of any supplementation of the medium, there was an overall decrease in the alpha diversity of the faecal microbiota after 24 hrs *in vitro* fermentation, measured here by the Shannon diversity index. The decrease in the microbiota diversity was expected due to the adaptation to the *in vitro* conditions and it was in line with published *in vitro* studies that reported that the microbiota diversity decreased over the first fermentation hours and began to increase after 72 hrs without attaining the diversity level of the initial stool sample (Rajilic-Stojanovic *et al*., 2010). Here, we aimed to observe which of the supplementation regimes sustained the highest microbiota diversity. The effect on faecal microbiota by medium supplementation is shown in **Figure 6 A** aggregated across all six donors for each growth medium; the diversity of the respective 0 hrs sample, and the diversity of the initial stool are also shown in **Figure 6 A**. The mean Shannon diversity index value at time-point 0 hrs was 3.4 (±0.6 SD). After 24 hrs fermentation with basal medium (no carbohydrate supplementation), supplemented with glucose or with lactose, the diversity decreased to a Shannon diversity value of ca. 2.4. Fermentation with medium supplemented with GMP resulted in higher diversity values. Supplementation with CGMP-10, CGMP-10 combined with glucose, and supplementation with tGMP resulted in Shannon diversity scores of 3.0 (±0.4 SD), 2.7 (±0.6 SD) and 2.8 (±1 SD) respectively. Wilcoxon test of ranking of the Shannon diversity index of paired individual samples by medium supplementation and time (between 24 hrs and 0 hrs) showed that after lactose fermentation, the faecal microbiota alpha diversity decreased significantly ($p=0.03$) compared to 0 hrs. There was a stronger trend in the decrease of Shannon diversity after fermentation with basal medium or medium supplemented with glucose, or with

CGMP-10 combined with glucose (p=0.06) compared to the decrease in Shannon diversity after fermentation with CGMP-10 supplemented medium (p=0.16). Similar effect of the substrates on the faecal microbiota diversity was observed on aggregated community samples, and aggregated longstay type samples, where the CGMP-10 supplemented medium sustained higher microbiota diversity compared to the other substrates tested (**Figure 6 B**).

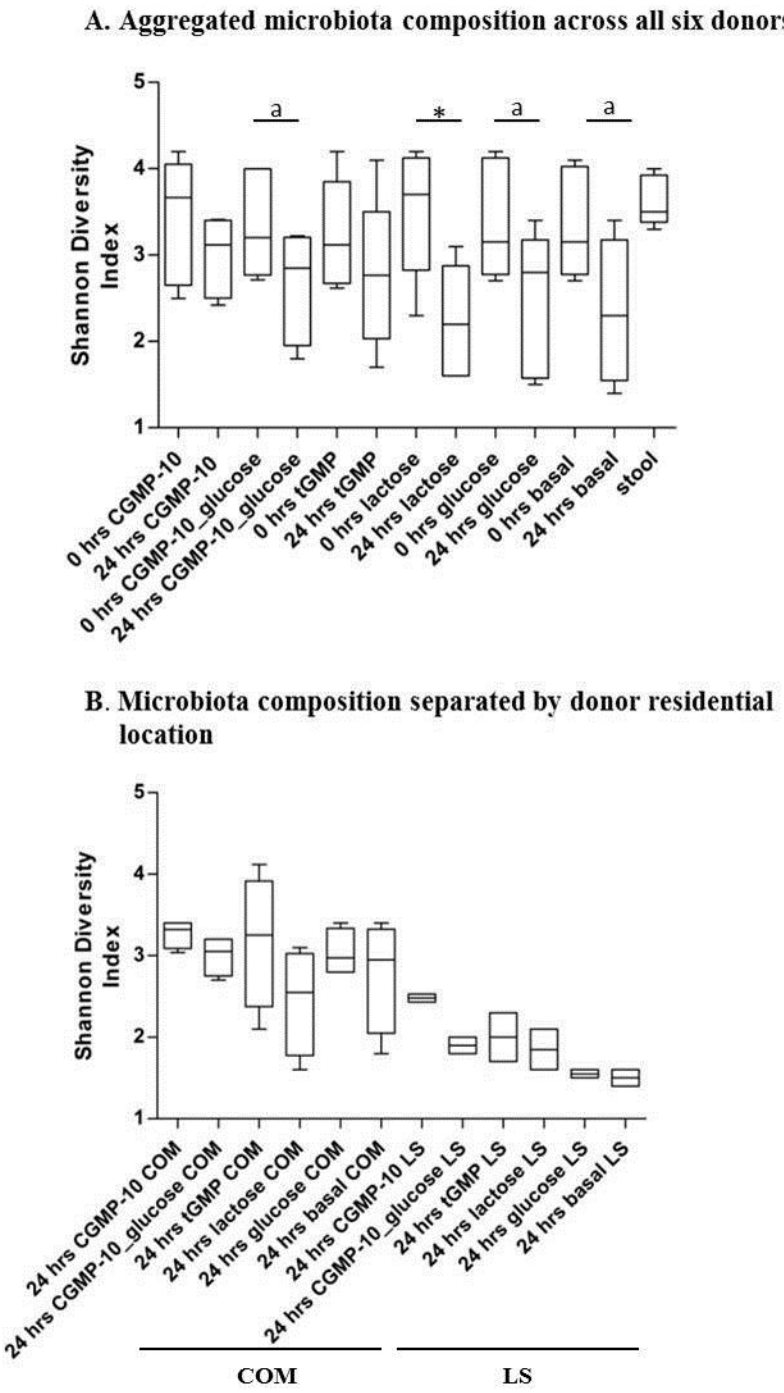


Figure 6 Effect of the fermentation substrates on the alpha diversity (Shannon diversity index) of the faecal microbiota. Effect on diversity of aggregated microbiota composition across all donors (**A**) and microbiota separated by donor residential location, i.e. community (COM) and longstay (LS) (**B**). Stool sample represents the faecal microbiota before the preparation of inoculum faecal slurry. Wilcoxon test result: "*" $p<0.05$; "a" $p<0.1$.

Detailed microbiota responses to GMP supplementation

Community type microbiota: Although retention of microbiota diversity upon supplementation with GMP is desirable, it was necessary to examine fine-detail microbiota effects. The microbiota profile at family and genus level for community aggregated samples is shown in **Figure 7 A** and **B**. The baseline high proportion of Lachnospiraceae was maintained after fermentation supplemented with all substrates tested (except after basal medium fermentation) and predominantly with CGMP-10 combined with glucose (average 51% relative abundance). Supplementation of the fermentation medium with CGMP-10 had a unique effect on the relative abundance of the families Rickenellaceae and Acidaminococcaceae that increased from an average 1.8% abundance to 3% and 4.5% respectively. Supplementation of the fermentation medium with CGMP-10 supressed the growth of Erysipelotrichaceae compared to supplementation with the other substrates as shown in **Figure 7 A**. The lowest proportion of the family Bacteroidaceae was observed after lactose fermentation (average 4% relative abundance) whereas after fermentation with the other tested media the relative abundance of the family remained similar to baseline (approximately 10%). High Enterobacteriaceae relative abundance increase was observed after fermentation with basal medium from an average 0.8% relative abundance at baseline to an average 30%. Substantial increase in the relative abundance of Enterobacteriaceae was also observed after fermentation with lactose or tGMP supplemented medium. In both supplementation regimes, i.e. lactose or tGMP, the proportion of the Enterobacteriaceae increased to approximately 17% relative abundance. The Enterobacteriaceae affected comprised mainly of the genera *Escherichia/Shigella* (**Figure 7 B**). Clostridiaceae 1 (comprised of the *Clostridium* sensu stricto cluster) relative abundance demonstrated an increasing trend regardless

of fermentation substrate (**Figure 7 A**). After basal medium fermentation Clostridiaceae 1 relative abundance had the highest increase compared to the other substrate fermentations, increasing from approximately 1.6% relative abundance at baseline to 13%. Fermentation with GMP-supplemented medium resulted in >6.5% relative abundance of Clostridiaceae 1.

Longstay type microbiota: In fermenters seeded with the longstay type microbiota the increase in relative abundance of the Clostridiaceae 1 and Enterobacteriaceae families was the predominant observation irrespective of the fermentation medium suggesting loss of major taxa in the faecal microbiota under 24 hrs *in vitro* conditions (**Figure 7 A**). The dominant genera were *Clostridium* sensu stricto and *Escherichia/Shigella* respectively (**Figure 7 B**). The proportion of the relative abundance occupied by the families Clostridiaceae 1, Enterobacteriaceae and Peptostreptococcaceae exceeded 60% across the samples. The predominant group in Peptostreptococcaceae was *Clostridium* cluster XI (**Figure 7 B**). Interestingly, although fermentation supplemented with CGMP-10 resulted in a high combined relative abundance of the families Clostridiaceae 1, Enterobacteriaceae and Peptostreptococcaceae (approximately 70%) there was also an increase in the relative abundance of Lachnospiraceae from approximately 4% to 11%, and maintenance of the low relative abundance of Streptococcaceae (<0.5%). Fermentation supplemented with lactose also resulted in increased Lachnospiraceae relative abundance (to approximately 7.5%) but lactose also promoted the growth of Streptococcaceae from <0.5% relative abundance to approximately 20%.

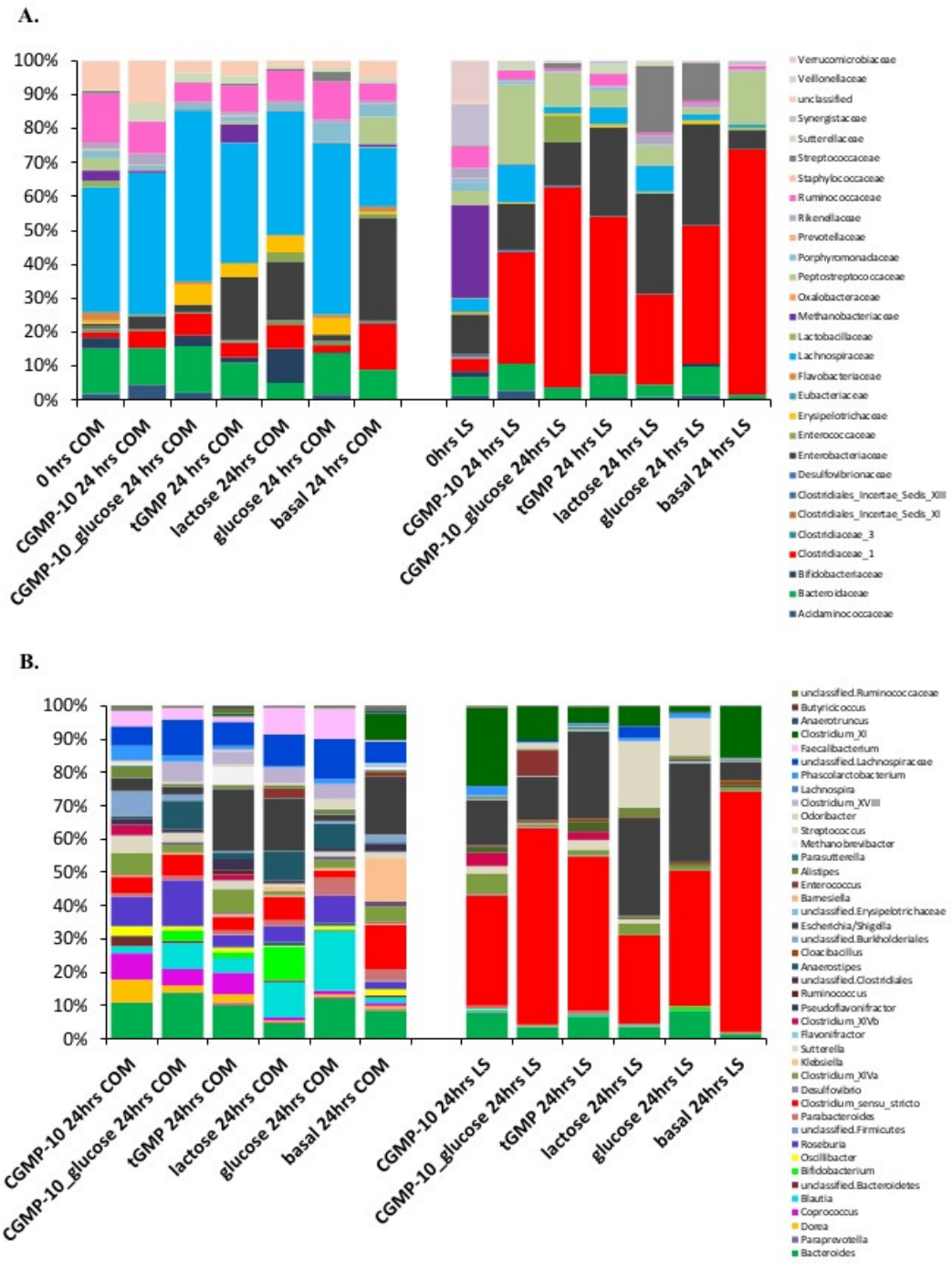
CGMP-10 positively affected the growth of health-related taxa, predominantly in community type microbiota

To provide further resolution we used the DESeq2 algorithm to identify bacterial taxa showing altered abundance after 24 hrs fermentation supplemented with the selected substrates (**Figure 8**). For certain taxa, change in the abundance after 24 hrs fermentation was similar irrespective of the fermentation supplement. The abundance of *Clostridium* cluster IV, *Ruminococcus* and *Lachnospira* decreased in both faecal microbiota types irrespective of fermentation supplement. With one exception (fermentation of glucose-supplemented medium by longstay type microbiota) *Sutterella* abundance increased in both microbiota types after fermentation with all fermentation supplements. *Clostridium* cluster sensu stricto and *Escherichia/Shigella* abundance also showed an increasing trend irrespective of fermentation supplement.

Supplementation with CGMP-10 of the artificial colon medium seeded with either microbiota type led to an increase of *Clostridium* cluster XIVb and *Coprococcus* abundance. In the community type microbiota the increase was approximately 6-fold (padj<0.0005) and 3-fold (padj=0.05) respectively. In the longstay type microbiota the respective fold-changes were approximately 10 (padj<0.0005) and 1 (not significant). An increasing trend in the abundance of *Pseudoflavonifractor* was observed after supplementation with CGMP-10; approximately 2-fold (not significant) and 16-fold (padj=0.08) increase in community and longstay microbiota respectively. After fermentation with medium supplemented with CGMP-10 by the community type microbiota an increasing trend in the abundance of the genus *Roseburia* by approximately 6-fold (padj=0.08) was observed. The abundance of the genera *Dorea* increased by 8-fold without reaching significance. Importantly, analysis of the composition of the faecal microbiota per individual sample after fermentation with medium supplemented with CGMP-10 showed a consistent

increase of the relative abundnace of *Dorea* in all samples from healthy donors and EM703 (data not shown). Fermentations with CGMP-10 combined with glucose yielded similar changes (but not significant) in the abundance of the aforementioned taxa in the community samples as with fermentation with CGMP-10 alone. The tGMP also increased the abundance of *Clostridium* cluster XIVb; approximately 16-fold (not significant) in community type microbiota and 9 log2fold (padj<0.0005) in longstay microbiota.

For both microbiota types fermentation of lactose supplemented-medium yielded (mostly not significant) increase in the abundance of *Blautia* (longstay type; approximately 16-fold, padj<0.007), *Clostridium* cluster XVIII (community type; approximately 64-fold, padj<0.06), *Enterococcus*, *Streptococcus*, *Escherichia/Shigella*. Increase in *Bifidobacterium* abundance was observed only when baseline abundance was high as in the case of EM278 (data not shown).



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3566 **Figure 7 Faecal microbiota profile after 24 hrs fermentation.** Community type

3567 (COM) and longstay type aggregated (LS) microbiotas are presented at family level

3568 (**A**) and genus level (**B**).

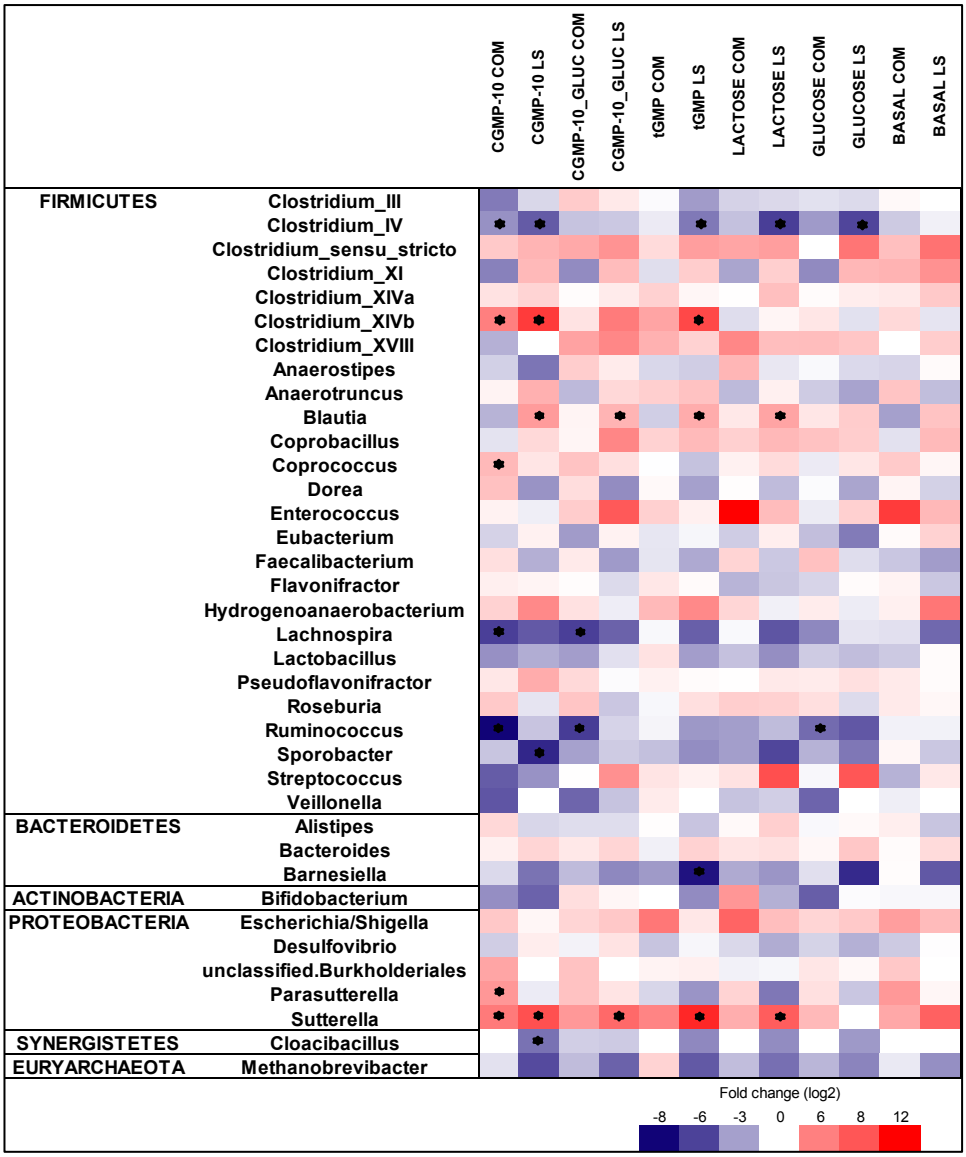

Figure 8 Heat-map of the changes in bacterial genus abundance in the faecal microbiota after 24 hrs fermentation with selected substrates. Comparisons were performed pairwise between 0hrs and 24 hrs for aggregated microbiota types by residential location as defined in main text. The logarithmic (log2) fold change results were generated using the DeSeq2 package. COM and LS correspond to community type and longstay type aggregated faecal microbiotas respectively. Significance is denoted by "*" for p values adjusted for multiple testing using Benjamini-Hochberg, padj<0.05.

GMP supplementation and SCFA production in the faecal microbiota of healthy elderly subjects

Community type faecal microbiota fermentation with GMP supplementation (substrate conditions iv, v, vi as shown in Materials and Methods) resulted in higher SCFA (i.e. propionate, acetate, butyrate) production compared to the other media tested; GMP-supplemented media: >148μmol of propionate, acetate and butyrate combined per grams of faecal pellet, lactose supplemented medium: mean of 113.3 μmol combined SCFA per g of faecal pellet, glucose supplemented medium: mean of 133.7 μmol combined SCFA per g of faecal pellet, basal medium: mean of 66 μmol combined SCFA per g of faecal pellet (**Figure 9 A**). No significant differences were obtained when comparing the SCFA production after supplementation with either GMP supplementation regime or lactose against the SCFA production after fermentation with medium supplemented with glucose; only fermentation with basal medium resulted in significantly lower production of propionate ($p<0.05$) and butyrate ($p<0.005$). For the longstay type microbiota the profile of the SCFA production was similar irrespective of the fermentation substrate (**Figure 9 B**) mirroring the loss of major taxa, among them potentially butyrate producers, observed for the profile of the microbiota composition (**Figure 7**). No statistical analysis was performed for longstay microbiota SCFA production due to the small group size.

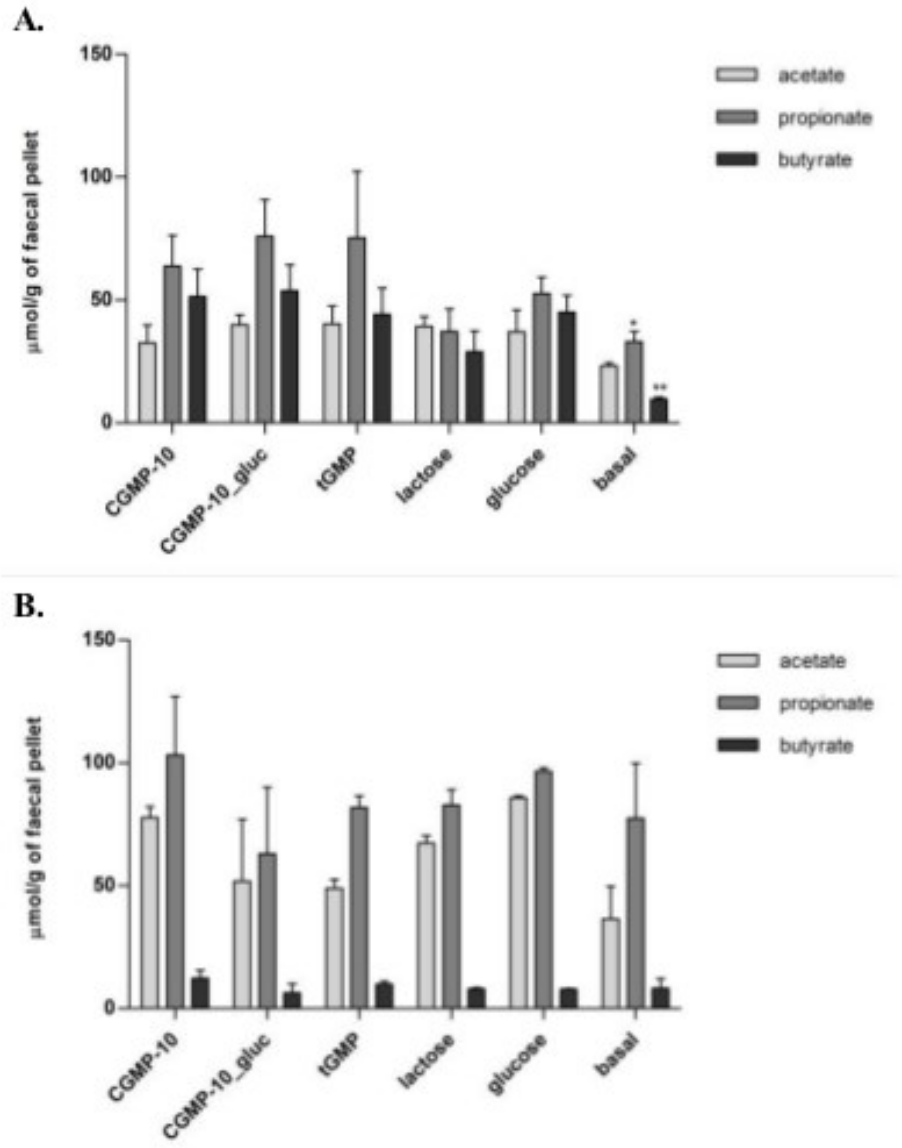

Figure 9 Short chain fatty acid (SCFA) levels in the faecal microbiota after 24 hrs fermentation in media supplemented as indicated. Concentration (µmol/g of faecal pellet) of acetae, propionate and butyrate in aggregated community (A) and longstay (B) microbiotas. Kruskal-Wallis test with Dunn's post hoc test was performed to compare SCFA production at 24 hrs after supplementation with the various substrates against glucose supplementation in the community type microbiota; "*" $p<0.05$, "**" $p<0.005$.

2.5 Discussion

Prebiotic effect of GMP on the faecal microbiota of healthy older donors

We investigated the *in vitro* effect of GMP supplementation on the gut microbiota using two GMP preparations and two types of faecal microbiota, i.e. community type and longstay type. CGMP-10 had a lower concentration of lactose compared to tGMP. The two microbiota types had major composition differences summarised by reduced overall diversity and abundance of Firmicutes, enrichment in Euryarchaeota, Proteobacteria and proteolytic taxa like Synergistetes and Verrucomicrobia in the longstay compared to the community type. The distinctive longstay microbiota profile apparently explains the loss of major taxa and the dominance of *Clostridium* sensu stricto cluster that was observed after fermentations with all tested substrates, because ecosystem stability and adaptive responsiveness to environmental changes are known to increase with phylogenetic diversity (Cadotte *et al*., 2012; Salonene *et al*., 2014; Alberdi *et al*., 2016).

For both microbiota types, supplementation of the fermentation medium with CGMP-10 sustained a higher diversity microbiota compared to the other media followed in rank order by the other two GMP-containing media, ie. tGMP and CGMP-10 combined with glucose. GMP containing medium resulted in higher total SCFA production from community microbiota-seeded fermentations compared to the other substrates tested. Increased SCFA concentration in the colon is known to correlate with a wide portfolio of health benefits (Koh *et al*., 2016).

The Lachnospiraceae member *Coprococcus* spp. is one of the health-relevant taxa that was positively affected by CGMP-10 supplementation as shown by the increase in the relative abundance of this group from the starting levels in the faecal

microbiota of mostly healthy donors after 24 hrs fermentation. In our previous studies, low abundance of coprococci in the faecal microbiota of older donors correlated with poor health status and a less diverse diet that was low in fibre (Claesson *et al*., 2012; Jeffery *et al*., 2016). Based on studies of the faecal microbiota from patients with colorectal cancer increased relative abundance of *Coprococcus* was negatively correlated with the condition (Flemer *et al*., 2016). CGMP-10 had a positive effect on the abundance of *Dorea* in the faecal microbiota of community dwelling older subjects. The increase in abundance of these two taxa indicates potentially important health benefits deriving from the consumption of GMP because *Coprococcus* and *Dorea*-enriched gut microbiota was found to be significantly resistant to pathobiont colonisation (Kampmann *et al*., 2016). CGMP-10 also positively affected the abundance of *Clostridium* cluster XIVb in both sample types. In a murine model of leukaemia and cachexia, cluster XIVb and *Parasutterella* species were among the first taxa to reduce in abundance in the murine faecal microbiota in these two diseases (Bindels *et al*., 2016). In this study the abundance of *Parasutterella* in the community type microbiota significantly increased after CGMP-10 supplementation. Importantly, *Clostridium* cluster XIVb is among the bacterial groups that increase in abundance after restoration of dysbiotic *Clostridium difficile*-associated diarrhoea gut microbiota by faecal microbiota transplantation (Brown *et al*., 2016). CGMP-10 had a positive effect on the abundance of *Roseburia* but only for the community type microbiota. For this taxon it is well-documented that it contributes to butyrate production which is a major contributor for colonic health (Koh *et al*., 2016). The differential response may be due to the low starting abundance levels of the genus *Roseburia* in the longstay type microbiota.

Lactose content of supplemented medium increased Proteobacteria abundance

Lactose-dependent modulation of the gut microbiota resulted in increased relative abundance of taxa related to inferior health status. Both lactose and tGMP enhanced the growth of Enterobacteria of the phylum Proteobacteria. This Phylum harbours pathobionts like the facultatives *Escherichia*/*Shigella* that can lead to disease (Kamada *et al*., 2013). Lactose fermentation promoted the increase in abundance of the Erysipelotrichaceae *Clostridium* cluster XVIII. This taxon has been associated with inferior health status in older subjects residing in long-term care units (Jeffery *et al*., 2016). In this study we could not conclude on the prebiotic potential of lactose. Other studies have reported a positive effect of lactose on lactobacilli and bifidobacteria (Szilagyi, 2015). *In vivo* trials will be conducted to further elucidate the effect of lactose on the faecal microbiota and to allow for lactose-related dietary recommendations.

Effect of the *in vitro* conditions on the faecal microbiota

In this study faecal samples were treated individually as opposed to samples being pooled from several donors, an approach used in some other studies (Aguirre *et al*., 2014). We opted for this because pooling faecal sample produce inocula that would be non-representative of average "real" microbiota (Payne *et al*., 2012). Partly in agreement with previous studies (Rajilic-Stojanovic *et al*., 2012), here we report decrease of microbiota diversity and decrease in abundance of *Clostridium* cluster IV as a result of all of the *in vitro* conditions rather than a result of any individual fermentation substrate. A decrease in the abundance of *Ruminococcus* was also observed irrespective of fermentation substrate or microbiota type and potentially due to the luck of resistant starch in the fermentation media (Ze *et al*., 2012).

Omitting resistant starch was done to avoid missing the effects conferred by the carbohydrates attached to the GMP. Interestingly, increase in *Blautia* spp. was observed in most longstay samples irrespective of fermentation medium suggesting a potential underlying longstay-type related trend. Bacteria of the genus *Blautia* are a prominent member of the healthy adult gut microbiota (Touyama *et al*., 2015) and are reduced in abundance in older individuals (Claesson *et al*., 2012). In spite of the predominant role of this taxon in the "healthy" adult microbiota, increased abundance of *Blautia* spp. can correlate to colorectal cancer (Flemer *et al*., 2016). For this reason the health benefit of promoting their growth by prebiotics is debatable, at least until species detail is available.

In search for new potential prebiotics we demonstrate here the capacity of GMP, a milk derivative with documented anti-inflammatory properties, to modulate *in vitro* the gut microbiota of elderly subjects by promoting the growth of several health-relevant taxa. GMP also increased SCFA production and sustained the diversity of the microbiota from healthy elderly inocula and to a lesser extend in inocula from frail elderly under *in vitro* conditions. *In vivo* trials are necessary to confirm the microbiota programming by GMP observed here since *in vitro* systems present certain limitations in simulating the real-life effect of diet compounds on the gut microbiota. [49] Importantly, since there are indications that inferior gut microbiota diversity may be associated with frailty in older age (O'Toole and Jeffery, 2015) GMP could contribute to amelioration of frailty scores by promoting gut microbiota diversity.

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Chapter 3

Retention of microbiota diversity by lactose free milk-supplemented diet was comparable to soy protein diet in a mouse model of healthy and frail elderly gut microbiota

3.1 Abstract

Compositional and phylogenetic diversity shifts of the gut microbiota, i.e. dysbiosis, may lead to disease. Dysbiotic changes in the microbiota are observed during ageing. Manipulation of the gut microbiota using prebiotics is a therapeutic strategy towards improving dysbiosis. Milk is a source of nutrients including complex oligosaccharides (OS), the prebiotic potential of which remains unexplored. We used a murine model to explore the effect of milk products on high and low diversity faecal microbiota (from healthy and frail older subjects, respectively) in comparison to soy protein supplementation, a product known to promote microbiota diversity and health in mice.

The gut microbiota of conventional mice was eradicated using antibiotics; the mice were gavaged with microbiota from either a frail or a healthy donor. The mice received purified diets supplemented with either lactose free or whole milk, glycomacropeptide GMP, (a potentially prebiotic rich in sialic acid dairy product) or soy-protein. The gut microbiota was studied after a month of diet.

Lactose-free milk diet was as efficient as the control diet in retaining microbiota diversity and it retained significantly higher diversity than the other diets in mice transplanted with microbiota deriving from either human donor type. Whole milk was not as efficient as lactose-free milk in retaining microbiota diversity but the relative abundance of health relevant taxa like *Ruminococcus bromii* were increased. Glycomacropeptide supplementation alone led to significantly decreased diversity compared to lactose-free milk and previously reported prebiotic activity was not confirmed *in vivo*. Bacteroidetes and Enterobacteriaceae were highly responsive to GMP supplemented diet; the sialic acid content of GMP may partly explain this

microbiota response. When lacking lactose, the remaining carbohydrate component of milk sustained the growth of a wider range of microbiota members and promoted phylogenetic diversity in this murine model. Our results indicate the prebiotic potential of lactose-free milk.

3.2 Introduction

During the last decade, the bacterial component of the human gut microbiota has been extensively studied allowing for a more comprehensive understanding of the compositional and functional profile of the gut microbiota in health and disease (Marchesi *et al*., 2015). Predominantly the habitual diet and to a lesser extent host genetics are two shaping factors of the gut microbiota (Sonnenburg and Bäckhed, 2016; Kurilshikov *et al*., 2017; Rothschild *et al*., 2018). The term dysbiosis refers to shifts in the composition, function and phylogenetic diversity of the gut microbiota of non-healthy individuals in comparison to healthy controls (Manor *et al*., 2017). Metabolic disease, functional GIT disorders and cognitive disorders have been linked to dysbiosis (reviewed in Levy *et al*., 2017).

The low-grade chronic inflammation that characterises older age reflects the decline of immune system fitness to respond to stressors, a phenomenon called inflammageing (Franceschi and Campisi, 2014). Aberrant intestinal permeability and dysbiotic gut microbiota are potential inflammageing drivers (Franceschi and Campisi, 2014; Li *et al*., 2016; Thevaranjan *et al*., 2017). Gut microbiota dysbiosis can be further fuelled by the inflamed GIT environment (Zeng *et al*, 2016). For prevention and treatment of dysbiosis and associated conditions, therapeutic interventions based on probiotics, prebiotics or faecal microbiota transplants (FMT) are being developed (Scott *et al*., 2014).

Milk combines many nutrients including calcium, vitamin K, conjugated linoleic acid, fatty acids, complex sialylated oligosaccharides and glycoproteins (Rozenberg *et al*., 2016; O'Riordan *et al*., 2014). There is accumulating data pointing towards certain health benefits of milk and dairy consumption (Song *et al*., 2016; Larsson *et*

al., 2015; Rozenberg *et al*., 2016). Importantly, milk consumption may contribute to muscle and bone density maintenance in older consumers (Sahni *et al*., 2014; Lana *et al*., 2015). Despite the potential health benefits, studies have shown that elderly people often fail to meet current recommendations for daily dairy consumption partly due to misconceptions on lactose mal-absorption and dairy fat content (Power *et al*., 2014; Laird *et al*., 2016).

The effect of milk on the gut microbiota has not been extensively studied. A few recent studies have focused on the potential prebiotic effect of milk oligosaccharides on the gut microbiota. Charbonneau *et al*. (2016) using animal models "humanised" with infant gut microbiota showed that sialylated bovine milk oligosaccharides (BMO) promoted weight gain associated with *Bacteroides fragilis* and *Escherichia coli* responsiveness to BMOs under malnutrition conditions. Karav *et al*. (2016) showed that BMO released from glycoproteins could mimic human milk oligosaccharide (HMO) selectivity on *Bifidobacterium* strains in the infant gut microbiota. Boudry *et al*. (2017) observed that BMO-supplemented diet enhanced gut barrier function, increased caecal and colonic microbiota diversity and *Lactobacillus* relative abundance in a murine model of diet-induced obesity.

Glycomacropeptide (GMP) is a by-product of cheese making and it is released in whey protein from the kappa-casein fraction of milk by the activity of chymosin (Neelima *et al*., 2013). Glycomacropeptide has five mucin-type glycans, high content of sialic acid, high content of branched chain amino acids (BCAA) and essential amino acids (EAA) and it is depleted of aromatic amino acids (Neelima *et al*., 2013). Glycomacropeptide is associated with a number of health benefits including anti-inflammatory and anti-GIT pathogen activity, and it is currently being used as an

alternative source of protein for phenylketonuria (PKU) nutritional management (van Calcar and Ney, 2012; Hvas *et al*., 2016).

There is inconclusive data on the prebiotic potential of GMP. *Bifidobacterium* and *Lactobacillus* selective growth promotion by GMP has been reported based on tests in pure cultures but the selectivity failed to be confirmed when complex faecal microbiota communities were tested *in vitro* (Bruck *et al*., 2006; Hernandez-Hernandez *et al*., 2011; Robitaille, 2013; Ntemiri *et al*., 2017). Some *in vivo* studies showed that GMP administration increased the colonic abundance of *Lactobacillus* and *Bifidobacterium* (Hermes *et al*., 2013; Jimenez *et al*., 2017). Sawin *et al*. (2015) reported that a GMP-supplemented diet significantly decreased the caecal and colonic relative abundance of *Desulfovibrio* and Proteobacteria in wild type and PKU mice. Interestingly, GMP selectivity on promoting growth of Bacteroidetes taxa has also been reported (Sawin *et al*., 2015; Jimenez *et al*., 2017).

In this study, conventional mice were treated with a cocktail of antibiotics before receiving FMT from either a healthy or a frail older donor. The effect of a diet enriched in either whole milk, lactose-free milk or GMP on the faecal microbiota of older humans was then investigated in these "humanised" mice.

3.3 Materials and Methods

Animals and experimental design: The Health Products Regulatory Authority (HPRA) under the European Union Regulations authorised this project with authorisation number AE19130/P033. Nighty-six 6 week-old female and male C57BL/6 mice were purchased form Envigo RMS Ltd, Oxon, UK and caged in groups of six of the same sex. The mice were weighed during the first week and every subsequent week of the trial.

The timeline of the mouse trial is outlined in **Figure 10 A**. The mice acclimatised to the animal facility for one week before the commencement of the six weeks antibiotics (Abx) treatment for the depletion of the indigenous murine gut microbiota. Ampicillin (1g/L; Sigma Aldrich), Metronidazole (1g/L; Molekula), Vancomycin (500 mg/L; Molekula), Imipenem (250 mg/L; Molekula) and Ciprofloxacin-HCl (200 mg/L; Santa Cruz) were diluted in tap water and filtered sterilised before given to animals as described before (Bereswill *et al*., 2011). Animals had *ad libidum* access to Abx-water. Limited administration of HydroGel (ClearH2O.com) during the Abx treatment was used for the prevention of weight loss. One day wash-out with filtered tap-water followed the Abx treatment. Subsequently, the animals were "humanised" with FMT from either a healthy older donor or a frail older donor.

The mice were introduced to the experimental diets on the wash-out day. The duration of the dietary intervention was one month. Six male and six female mice were allocated to each diet **Supplementary** S **Table 1.** The animals were fed maintenance diet (ssniff, Spezialdiäten GmbH, German) during the acclimatisation period and during the Abx treatment. The experimental diets were isoenergetic and isonitrogenous refined maintenance diets (ssniff, Spezialdiäten GmbH, Germany) supplemented with one of the following powders: **i. lac-free diet:** 20% lactose-free whole milk powder (Valio Ltd., Helsinki, Finland), **ii. wmilk diet:** 20% whole milk powder (Valio Ltd., Helsinki, Finland), **iii. GMP diet:** 20% CGMP-10 (Arla Food Ingredients, Denmark), **iv. control diet:** 20% soy protein isolate. In order to achieve the isoenergetic and isonitrogenous profile of the diets, all diets were additionally supplemented with soy protein isolate that was the basic protein ingredient for the maintenance diet as shown in **Table 6**. The complete compositional content of the

experimental diets is attached in **Supplementary Material**. All diets were sterilized by irradiation.

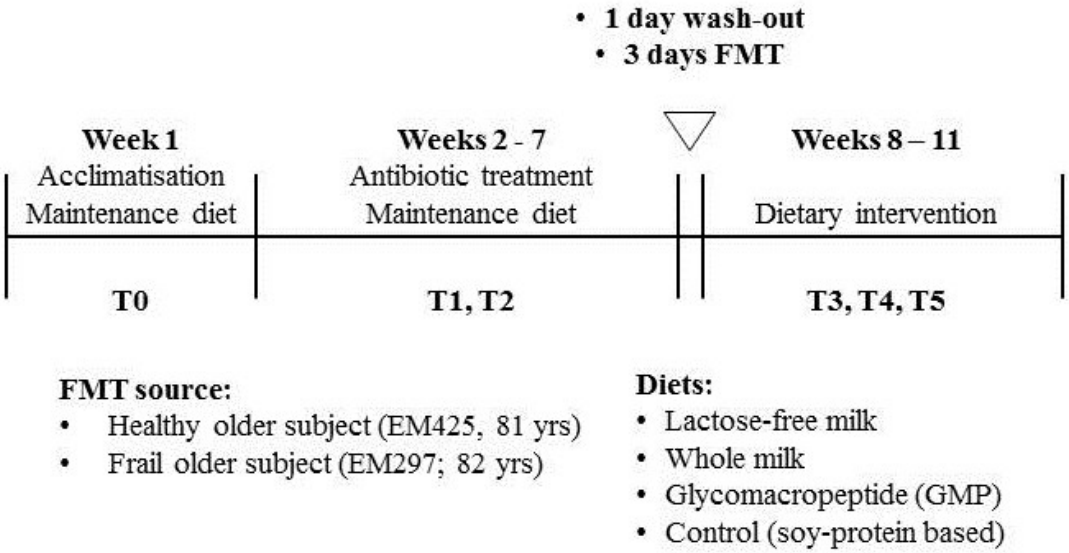


Figure 10 Timeline of the 11 weeks mouse trial. Time-points of samples collection: T0 during the acclimatisation, T1 and T2 during the Abx treatment, T3, T4 and T5 during the dietary intervention. The FMT source was used for the "humanisation" of the Abx- treated animals. The isocaloric and isonitrogenous diets were refined diets with 20% supplementation with the shown ingredients.

Table 6 Composition of the customized experimental diets (ssniff Spezialdiäten GmbH).

Ingredient %	Control	20 % whole milk powder	20 % lactose free milk powder	20 % CGMP-10 powder
Soy protein isolate	20.0	14.5	12.8	1.8
Whole milk powder	-	20.0	-	-
Lactose-free milk powder	-	-	20.0	-
Glycomacropeptide CGMP-10	-	-	-	20.0
Maltodextrin	33.1	33.2	32.9	32.0
Sucrose	10.5	2.5	4.3	10.3
Cellulose powder	6.0	4.9	5.0	5.2
Soybean oil	7.5	2.3	2.3	7.5
Proximate contents %				
Crude protein	18.1	18.1	18.1	18.1
Crude fat	7.6	7.6	7.6	7.6
Crude ash	4.6	5.4	5.6	5.5
Starch	31.8	31.9	31.6	30.8
Sugar (total)	12.3	12.3	12.3	12.3
Lactose	-	8.0	-	-
Energy (MJ/kg)	16.0	16.0	16.0	16.0
kcal % Protein	19	19	19	19
kcal % Fat	18	18	18	18
kcal % Carbohydrates	63	63	63	63

Faecal slurry preparation and mouse "humanisation": One faecal sample from a healthy 81 yr old subject (EM425; community type microbiota, COM) and one faecal sample from a frail 82 yrs old subject (EM297; longstay type microbiota, LS) were collected under the approval of the local Clinical Research Ethics

Committee. The samples were processed under anaerobic conditions for the preparation of 10 % w/v faecal slurries in PBS and 20 % glycerol. The faecal slurries were kept in aliquots at -80°C and thawed in the anaerobic cabinet before gavage to mice. The animals received by oral gavage a total of 300 µl of faecal slurry in three days.

Faecal and caecal sample collection: Faecal samples were collected during the trial at various time points (T0 to T5) as indicated in **Figure 10.** One to two faecal pellets were collected at each time point. Caecum content was collected at the end of the trial by dissecting the caecum and emptying the caecum content in sterile Eppendorf tubes. All samples were immediately frozen in liquid nitrogen before being transferred to -80°C.

Genomic DNA extraction: Genomic DNA was extracted from murine faecal pellets and caecum content using the QIamp Fast DNA Stool (Qiagen) kit. Murine faecal and caecal pellets were weighed before performing the extraction using the QIamp Fast DNA Stool (Qiagen) extraction kit protocol. The samples were placed in sterile tubes containing 0.1 mm, 0.5 mm and 1.0 mm zirconia / glass beads (Thistle Scientific, UK). Eight hundred ml of InhibitEX buffer was added to the samples and then homogenised under one pulse of 1 min and a final of 30 sec using a Minibeadbeader (Biospec Products). In the intervals of the homogenisation steps the samples were placed on ice for 1 min. The samples were then placed in a 95°C heat-block for 5 min. The subsequent steps of the DNA extraction were carried out as described in the Qiagen protocol using 15 µl of proteinase K with 200 µl of AL buffer, 200 µl of lysate and 200 µl of ethanol at the relevant steps. The final elution was performed at 70 µl of elution buffer.

Library preparation for 16S rRNA gene amplicon sequencing: The V3 / V4 variable region of the 16S rRNA gene was targeted for Illumina MiSeq System sequencing (San Diego, California, USA). The region was targeted and amplified using the universal 16S ribosomal RNA gene primers; forward primer for V3 region 5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWG CAG 3' and reverse primer for V4 region 5' GTCTCGTGGGCTCGGAGATGTG TATAAGAGACAGGACTACHVGGGTATCTAATCC 3' (Klindworth *et al*. 2012). The PCR products were purified and the subsequent library preparation steps were performed according to the Illumina MiSeq system protocol. Dual-index barcodes were attached to the amplicon (Nextera XT V.2 Index Kits sets A and D, Illumina) and then purified using the Agencourt AMPure XP-PCR Purification system (Beckman Coutler, Inc.). The indexed amplicons were quantified using the Qubit dsDNA HS Assay Kit (Thermo Fischer Scientific, MA, U.S.A.). The pooled library containing the same concentration of each amplicon was sequenced on a MiSeq Illumina platform using a 2 x 250 bp chemistry in the Teagasc Next Generation Sequencing Facility (Fermoy, Ireland).

Microbiota composition analysis and statistics: FLASH (Magoč *et al*., 2011) was used to join the paired reads that were subsequently demultiplexed in Qiime with the split_libraries.py script and forward and reverse primers were removed from the reads. Usearch was used for the *de novo* generation of an operational taxonomic unit (OTU) table. The reads were filtered by read length and the number of identical sequences, before chimera filtering was applied. Subsequently, all reads were mapped against the representative reads generated by the filtering based on 97% identity. The reads per sample were rarefied at a number of reads in order to include the majority of the samples collected throughout the

study. The sequences were aligned using the PyNast tool (Caporaso *et al*., 2010) in Qiime in order to generate alpha diversity indices i.e., Shannon, Simpson, PD whole tree, Chao1 and observed species, and beta diversity indices i.e. Weighted UniFrac and Unweighted UniFrac. The *de novo* OTU table generated based on the filtered reads was taxonomically classified (classify.seqs) down to genus level by using Mothur and the RDP v11.4/trainset 14 database (Wang *et al*., 2007). The Spingo database was used in order to classify the reads at species level (Allard *et al*., 2015). The software R (R Core Team) was used in order to convert the reads per sample into relative abundances, to analyse and visualise the UniFrac distances, and to visualise the alpha diversity of the microbiota. A Kruskal-Wallis with Dunn's post hoc test with p adjusted value with the Benjamini-Hochberg method were applied in R in order to detect significant differences in the faecal microbiota diversity due to diet at various time points. A Kruskal-Wallis with Dunn's post hoc test with p adjusted value with the Benjamini-Hochberg method was applied in order to detect the differentially abundant taxa in the aggregated microbiota of female and male mice at the end of the trial as a result of the different diets. Values were considered significant when Kruskal-Wallis p value was ≤ 0.5, p_{adj} value ≤ 0.05 and Dunn's post hoc test p_{adj} values was ≤ 0.05.

3.4 Results

Milk supplemented diets resulted in the highest increase in body weight from baseline

Due to severe weight loss during the first 2 weeks of Abx treatment, 6 animals were euthanized (**S Table 1**). Some animals were given HydroGel for up to 9 days (**S Table 1**) in order to prevent further weight loss due to a version of the mice to the

Abx-containing water and subsequent abstaining from food. All animals began to recover their body weight after the 2nd week of Abx treatment (**Figure 11**) and at the end of the trial the diets had a differential effect on weight gain compared to the baseline body weight (**Figure 11**). Lactose-free milk supplemented diet resulted in the highest increase in body weight in mice humanised with COM type microbiota (**Figure 11 A**), while whole milk diet resulted in the highest increase in weight in mice humanised with the LS type microbiota (**Figure 11 B**). Upon dissection all animals had normal spleen weight (data not shown).

Differences between the human donors and the murine baseline faecal microbiota composition

Compositional and phylogenetic diversity differences in the faecal microbiota of frail long-term care unit-residing elderly subjects and healthy community-living were described in the ElderMet study (Claesson *et al*., 2012). The ElderMet donors EM297 (LS) and EM425 (COM) were re-enrolled and their faecal microbiota was re-analysed in order to confirm suitability for the mouse trial. The compositional differences between the faecal microbiotas from subjects EM297 (LS) and EM425 (COM) have been partly described in Chapter 2 (Ntemiri *et al*., 2017). Compared to the COM type microbiota, the LS had lower Firmicutes relative abundance, it was enriched in Proteobacteria, Synergistetes and Bacteroidetes, whereas the alpha diversity was lower (**Figure 12**, **S Tables 2 and 3**). Thus, the inocula were considered suitable for proceeding with mouse humanisation.

The composition of the murine faecal microbiotas is shown in **Figure 12** (**S Table 2).** In the murine faecal microbiota, the phylum Bacteroidetes was the prevalent phylum (56 % average relative abundance) followed by the Firmicutes. Compared to

4094 the human microbiota, the murine microbiota was enriched in certain Bacteroidia
4095 including *Barnesiella intestinihominis*, *Alistipes massiliensis*, unclassified
4096 Prevotellaceae and Porphyromonadaceae (**S Table 2**). Among the Firmicutes, taxa
4097 that belonged to *Lactobacillus* and unclassified Lachnospiraceae were dominant in
4098 the murine faecal microbiota. The alpha diversity of the baseline murine microbiota
4099 was comparable to the COM type microbiota alpha diversity (**S Table 3**).

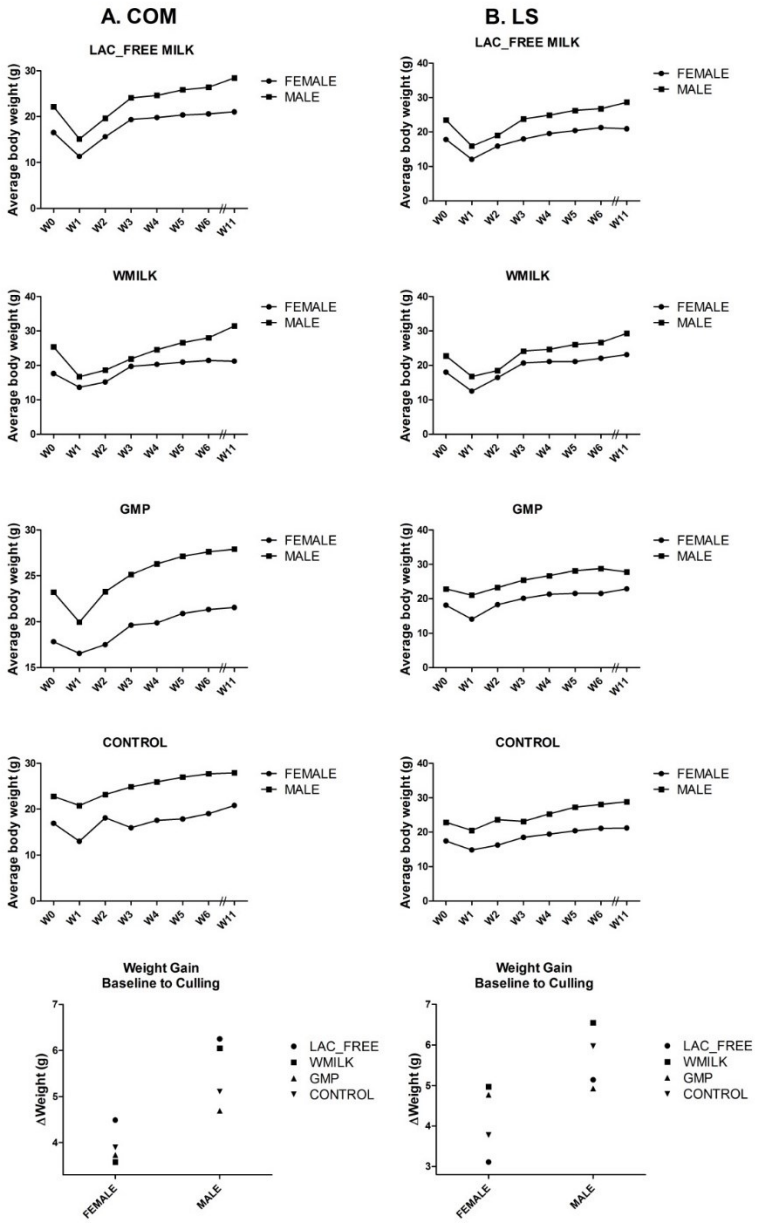


Figure 11 Animal body weight during the 11 weeks of the trial. The body weight from the first week of Abx treatment (W0) till the completion of the trial (W11), is shown. Body weight change between baseline and end of trial is shown at the bottom. **A** and **B**: COM: community type humanisation and LS: longstay type humanisation, respectively.

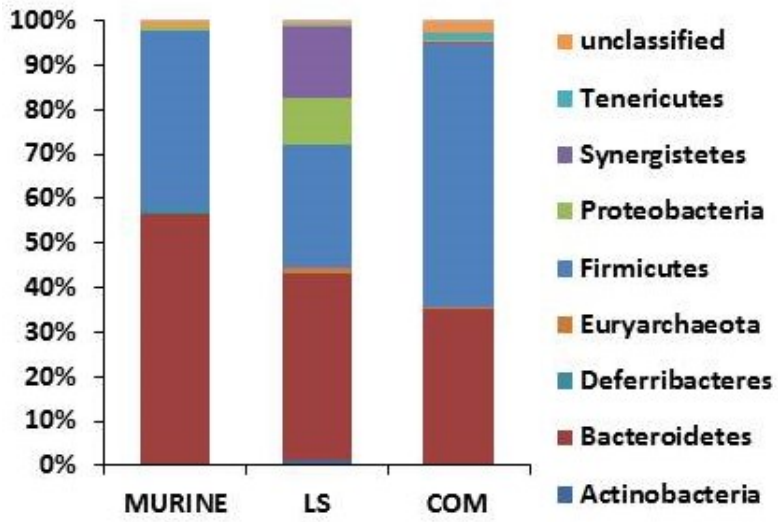


Figure 12 Composition of the human and murine baseline faecal microbiota at phylum level. Phyla that were present at ≥1 % relative abundance are presented. LS: longstay type faecal microbiota; COM: community type faecal microbiota; murine: aggregated faecal microbiota across all mice at baseline.

Efficiency of colonisation of the murine gut by the human faecal microbiota

One week after humanisation (T3), the murine faecal microbiota had diverged from baseline and did not revert back to the murine phylogenetic profile by the completion of the trial as demonstrated by β diversity UniFrac analysis throughout the trial (**S Figure 1, 2)**. Importantly, the COM or LS phylogenetic profile was retained throughout the trial as shown by principal coordinates analysis (PCoA) of the Unweighted UniFrac distances at the end of the trial (T5) in spite of the strong effect of diet (**S Figure 1 B** and **2 B**).

Lactose-free milk supplemented diet sustained significantly higher alpha diversity faecal microbiota compared to the other diets in both community and longstay type humanised mice.

Community type microbiota: There was no difference in the effect of lactose-free diet and control diet on the microbiota diversity. In contrast the GMP diet resulted in the lowest retention of alpha diversity across all indices (**Figure 13**). Lactose-free milk, control and whole milk diets resulted in significantly higher Shannon, Simpson and PD indices compared to GMP diet. For the indices Observed species and Chao1, lac-free and control diet retained significantly higher faecal microbiota diversity compared to both wmilk and GMP diet. A detailed summary of the statistical analysis results is shown **S Table 4**.

Longstay type microbiota: The lactose-free milk diet intake resulted in significantly higher Shannon and Simpson diversity indices compared to all diets including control (**Figure 14)**. The lactose-free diet intake resulted in significantly higher PD whole tree index compared to the whole milk and GMP diet intake whereas alpha diversity scores after control diet were significantly higher only compared to GMP diet. The lactose-free and control diet resulted in significantly higher Observed Species index compared to the whole milk and GMP diets. A detailed summary of the statistical analysis results is shown **S Table 5**.

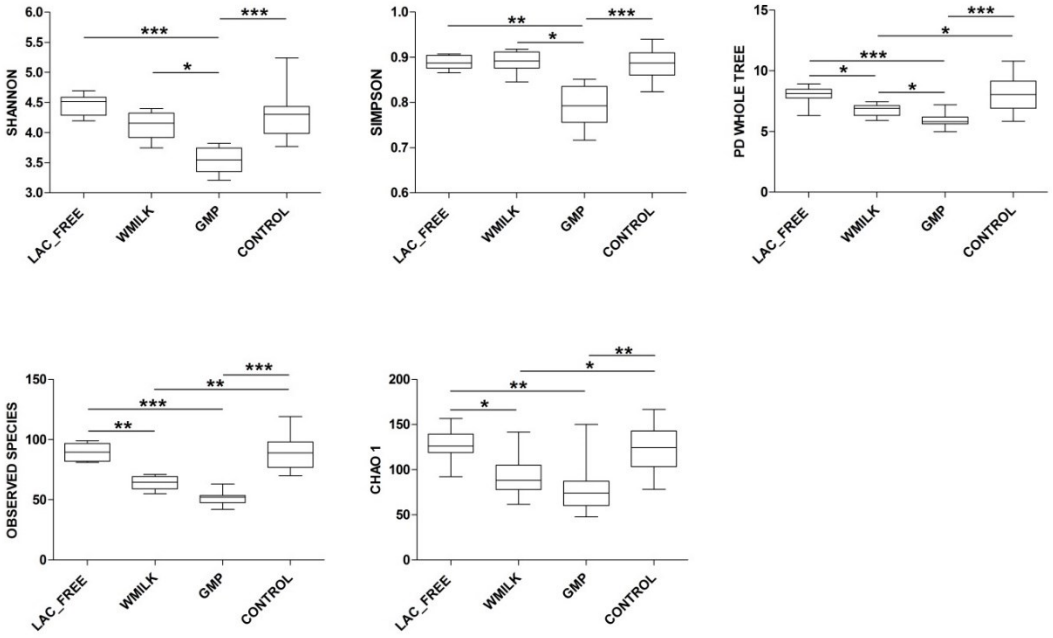


Figure 13 Alpha diversity indices at the end of the trial (T5) for mice humanised with community (COM) type faecal microbiota. LAC_FREE: lactose-free milk supplemented diet, WMILK: whole milk supplemented diet, GMP: glycomacropeptide supplemented diet, CONTROL: soy-protein based control diet. Only tests that passed the $p_{adj} \leq 0.05$ threshold for the Kruskal Wallis test are presented. Asterisks refer to the Dunn's post hoc test: * $p_{adj} \leq 0.05$, ** $p_{adj} \leq 0.005$, *** $p_{adj} \leq 0.0005$. Details on the values are given in S Table 4.

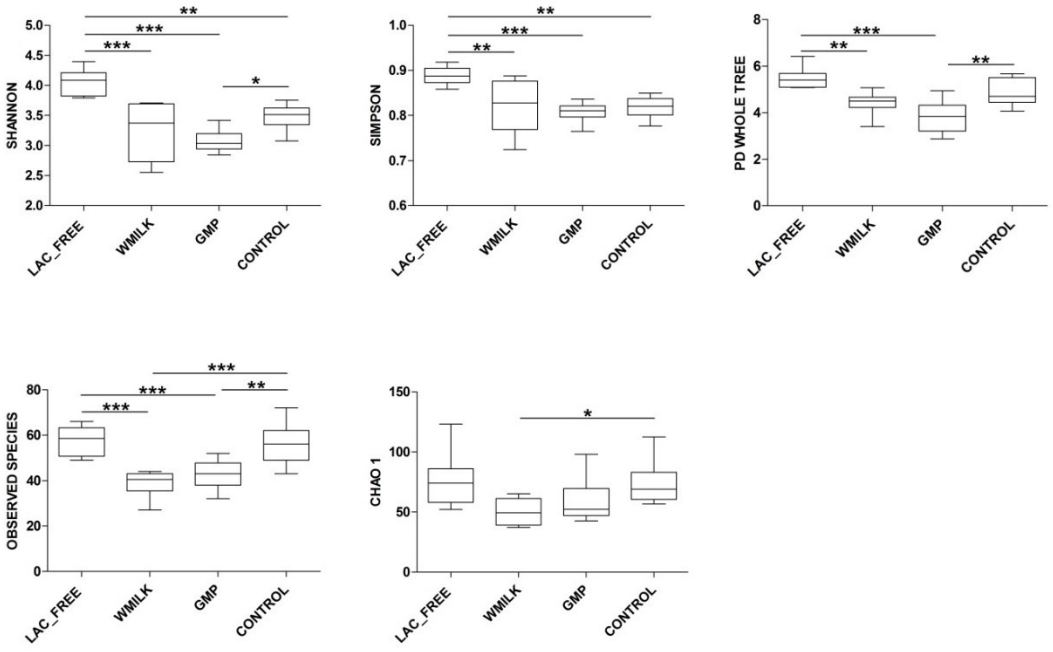


Figure 14 Alpha diversity indices at the end of the trial (T5) for the faecal microbiota of mice humanised with longstay (LS) type faecal microbiota. LAC_FREE: lactose-free milk supplemented diet, WMILK: whole milk supplemented diet, GMP: glycomacropeptide supplemented diet, CONTROL: soy-protein based control diet. Only tests that passed the $p_{adj} \leq 0.05$ threshold for the Kruskal Wallis test are presented. Asterisks refer to the Dunn's post hoc test: * $p_{adj} \leq 0.05$, ** $p_{adj} \leq 0.005$, *** $p_{adj} \leq 0.0005$. Details on the values are given in S Table 5.

Lactose-free milk diet sustained higher abundances of health relevant taxa including Ruminococcaceae compared to the other diets in mice humanised with community type microbiota.

At the end of the dietary intervention (T5) the faecal microbiota clustered based on diet; microbiota from mice fed all three dairy-supplemented diets clustered closer compared to microbiota from mice fed control diet as shown in **Figure 15** (Weighted PCoA of the UniFrac distances of the sequenced 16S rRNA gene sequences in the

relevant faecal microbiota samples). In the faecal microbiota of mice fed lac-free diet the Porphyromonadaceae was the most abundant family (41% average relative abundance) (**Figure 16**). The families Ruminococcaceae and Lachnospiraceae (17% and 15% average relative abundance respectively) were the next most abundant families in the microbiota composition of lactose-free diet fed mice; both families reached significantly high abundance upon lac-free diet compared to the other diets (**S Figure 3 A**). Other dominant families were the Bacteroidaceae, Desulfovibrionaceae and Rikenellaceae. (**Figure 16**). *Parabacteroides goldsteinii* was the most abundant taxon (28% average relative abundance) of the Porphyromonadaceae followed by *B. intestinihominis* and *Parabacteroides merdae* (**Figure 17**). *Anaerotruncus colihominis* and *Oscillibacter* taxa were the most abundant among the Ruminococcaceae (6% and 5% average relative abundance respectively) followed by *Flavonifractor plautii* (**Figure 17**). The Lachnospiraceae representatives belonged to unclassified taxa (**Figure 17**).

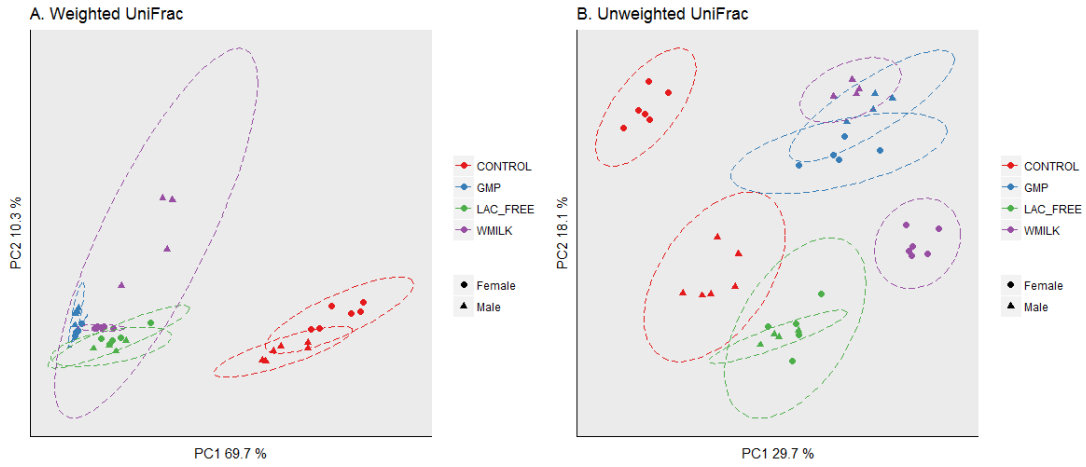


Figure 15 Principal coordinates analysis (PCoA) of the murine faecal microbiota at the end of the trial (T5). The mice were humanised with community (**COM)** type faecal microbiota. **A**: Weighted UniFrac distances; **B**: Unweighted UniFrac distances. LAC_FREE: lactose-free milk supplemented diet, WMILK: whole milk supplemented diet, GMP: glycomacropeptide supplemented diet, CONTROL: soy-protein based control diet."●" denotes female mouse microbiota; "▲" denotes male mouse microbiota.

Gender dependent gut microbiota response to whole milk in mice humanised with community type microbiota.

Based on the UniFrac analysis of the faecal microbiota of mice fed a whole milk supplemented diet, two sub-clusters were formed separating female and male microbiota responses (**Figure 15 A, B**). The Bacteroidaceae were more responsive to the wmilk diet in the female mouse faecal microbiota (23.6% and 6.5% average relative abundance in female and male mice respectively) whereas the Erysipelotrichaceae were more abundant in the male mouse microbiota (23% and 2% average relative abundance in male and female mice respectively) (**S Table 6**). *Bacteroides thetaiotaomicron* and *B. uniformis* were the predominant representatives of the Bacteroidaceae, and *A. stercorianis* and *Turicibacter sanguinis* were the major representative taxa of the Erysipelotrichaceae in male and female mouse microbiota respectively (**Table 7**). Gender based differences were also observed for the families Ruminococcaceae (more abundant in male) and Peptostreptococcaceae (more abundant in female) (**S Table 6**). *Ruminococcus bromii* (Ruminococcaceae) was sustained only by the whole milk diet in the microbiota of male mice (6% average relative abundance).

The compositional profile of the faecal microbiota aggregated across both genders is shown in **Figures 16** and **17**. Bacteroidaceae and Erysipelotrichaceae relative abundance was significantly increased upon whole milk diet compared to the other tested diets (**S Figure 3 A**). Compared to the lactose-free diet Porphyromonadaceae and Ruminococcaceae were less responsive to the whole milk diet whereas Rikenellaceae and Acidaminococcaceae were more responsive (**Figures 16; S Figure 3 A**). The Lachnospiraceae reached a relatively high abundance (11.7%

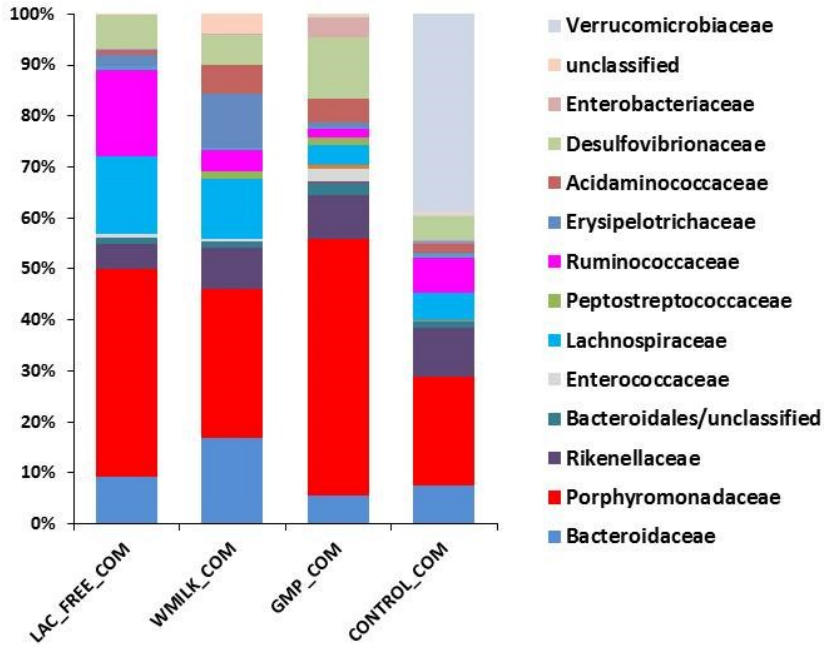
average relative abundance) similar to that after lac-free diet and compared to the other diets (**S Figure 3 A**).

Enrichment of the faecal microbiota in Porphyromonadaceae, Desulfovibrionaceae and potential pathobionts due to glycomacropeptide supplementation in community type humanised mice.

We have previously presented evidence of GMP prebiotic activity in an artificial colon model of elderly gut microbiota (Chapter 2; Ntemiri *et al*., 2017) but did not confirm these findings in our mouse model. The microbiota of both female and male mice responded similarly to the GMP diet as observed by the clustering on the PCoA analysis (**Figure 15 A** and **B**). Compared to the other diets, the GMP diet resulted in significant enrichment of the faecal microbiota in Porphyromonadaceae and the Desulfovibrionaceae (50% and 12% average relative abundance respectively) compared to most of the diets (**Figure 16; S Figure 3 A**). The main representative taxa of the two aforementioned families were *P. goldsteinii* and *B. wadsworthia* (46% and 12% average relative abundance respectively) (**Figure 17**). The GMP diet had, similar to the wmilk diet, an effect on the Acidaminococcaceae (5% average relative abundance) represented by *P. faecium* (**Figure 17**). Compared to the other diets, feeding with the GMP diet resulted in significant microbiota enrichment in Enterobacteriaceae and Enterococcaceae (4% and 2.5% average relative abundance respectively) (**Figure 16; S Figure 3 A**); *Enterococcus* and *Escherichia*/*Shigella* taxa were the dominant representatives (**Figure 17**).

Gender dependent gut microbiota response to control diet in community type humanised mice.

The faecal microbiota of mice fed the control diet clustered separately from the microbiota of mice fed dairy-supplemented diets and gender based sub-clustering was also observed (**Figure 15)**. Differences in the composition between female and male mice fed the control diet included differences in the abundance of taxa belonging to the Porphyromonadaceae, Rikenellaceae, Ruminococcaceae and Acidaminococcaceae (**Table 7**). Porphyromonadaceae was the next most abundant family (21% average relative abundance) in the microbiota of control fed mice following the Verrucomicrobiaceae (40% average relative abundance) represented by *A. muciniphila* (**Figures 16, 17**). The compositional profile of the faecal microbiota of mice fed the wmilk diet aggregated across both gender is shown in **Figures 16** and **17.**



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4246 **Figure 16 Composition of the murine faecal microbiota at family level at the**
4247 **end of the trial (T5) in mice humanised with community (COM) type human**
4248 **faecal microbiota**. LAC_FREE: lactose-free milk supplemented diet, WMILK:
4249 whole milk supplemented diet, GMP: glycomacropeptide supplemented diet,
4250 CONTROL: soy protein based control diet. Families present at ≥1 % are shown.

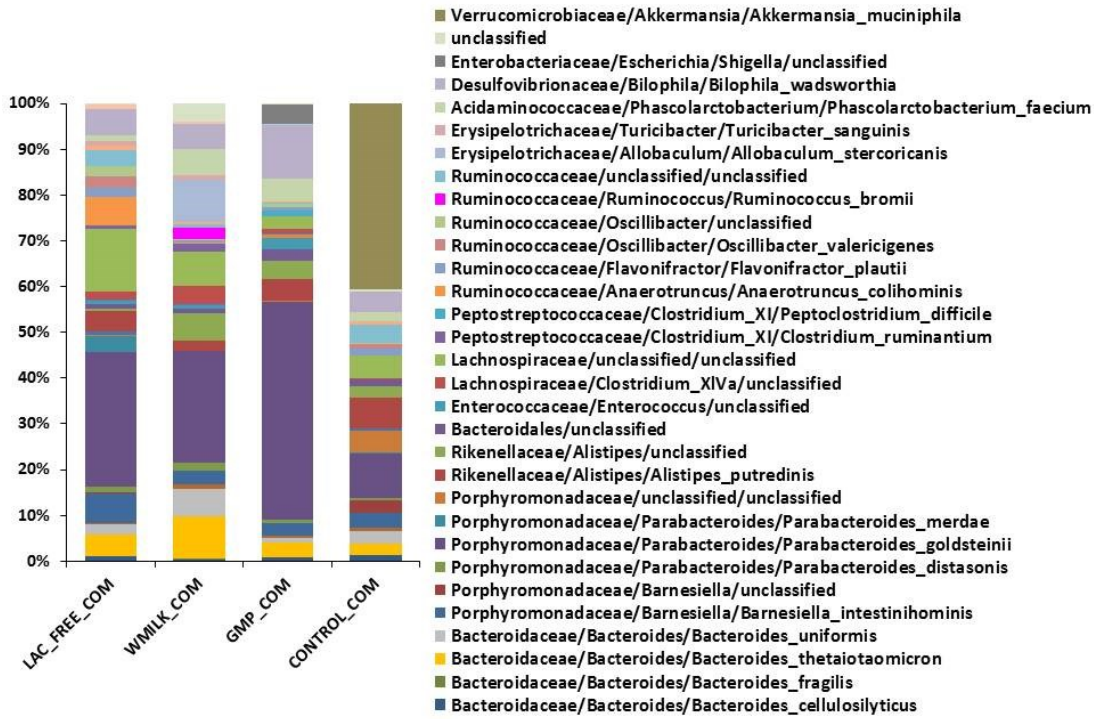

Figure 17 Composition of the murine faecal microbiota at species level at the end of the trial (T5) in mice humanised with community (COM) type human faecal microbiota. LAC_FREE: lactose-free milk supplemented diet, WMILK: whole milk supplemented diet, GMP: glycomacropeptide supplemented diet, CONTROL: soy protein based control diet. Only species present at ≥1 % are shown.

Table 7 Differences in the female and male murine microbiota composition associated with separation in the PCoA grouping by whole milk (WMILK) and control diet at the end of the trial (T5). Only species present at ≥1 % relative abundance are presented. The humanisation of the mice was performed with community (**COM**) type microbiota.

OTU classification	WMILK FEMALE	WMILK MALE	CONTROL FEMALE	CONTROL MALE
	% average relative abundance			
Bacteroidaceae/Bacteroides/Bacteroides cellulosilyticus	0.0	0.6	2.6	0.0
Bacteroidaceae/Bacteroides/Bacteroides thetaiotaomicron	14.1	1.3	2.3	2.3
Bacteroidaceae/Bacteroides/Bacteroides uniformis	7.7	2.7	1.7	4.0
Bacteroidaceae/Bacteroides/unclassified	1.3	0.4	0.2	1.0
Porphyromonadaceae/Barnesiella/Barnesiella intestinihominis	3.1	2.7	2.4	4.1
Porphyromonadaceae/Barnesiella/unclassified	0.1	0.04	4.7	0.2
Porphyromonadaceae/Butyricimonas/Butyricimonas virosa	0.5	1.3	1.0	0.4
Porphyromonadaceae/Parabacteroides/Parabacteroides distasonis	1.8	1.4	1.1	0.3
Porphyromonadaceae/Parabacteroides/Parabacteroides goldsteinii	24.5	22.0	6.0	12.7
Porphyromonadaceae/unclassified/unclassified	0.1	0.1	9.3	0.1
Rikenellaceae/Alistipes/Alistipes putredinis	0.01	5.4	0.01	12.6
Rikenellaceae/Alistipes/unclassified	6.5	4.2	0.4	4.7
Bacteroidales/unclassified/unclassified/unclassified	1.3	1.0	0.8	1.7
Lachnospiraceae/Clostridium XlVa/unclassified	6.4	0.2	0.002	0.6
Lachnospiraceae/unclassified/unclassified	4.1	12.3	5.2	4.5
Peptostreptococcaceae/Clostridium XI/ Clostridium ruminantium	2.7	0.0	0.0	0.0
Ruminococcaceae/Flavonifractor/Flavonifractor plautii	0.8	0.4	2.1	0.7
Ruminococcaceae/Oscillibacter/Oscillibacter valericigenes	0.0	0.02	1.6	0.2
Ruminococcaceae/Ruminococcus/Ruminococcus bromii	0.0	6.0	0.0	0.0
Ruminococcaceae/unclassified/unclassified	0.7	0.7	4.9	2.6
Clostridiales/unclassified/unclassified/unclassified	0.6	0.4	0.5	1.1
Erysipelotrichaceae/Allobaculum/Allobaculum stercoricanis	0.001	22.0	0.0	0.0
Erysipelotrichaceae/Turicibacter/Turicibacter sanguinis	1.6	0.0	0.0	0.001
Acidaminococcaceae/Phascolarctobacterium/ Phascolarctobacterium faecium	5.7	5.5	3.0	0.6
Desulfovibrionaceae/Bilophila/Bilophila wadsworthia	7.5	1.8	3.7	4.9
unclassified	5.3	1.5	0.9	0.2
Verrucomicrobiaceae/Akkermansia/ Akkermansia muciniphila	0.0	0.0	40.6	37.2

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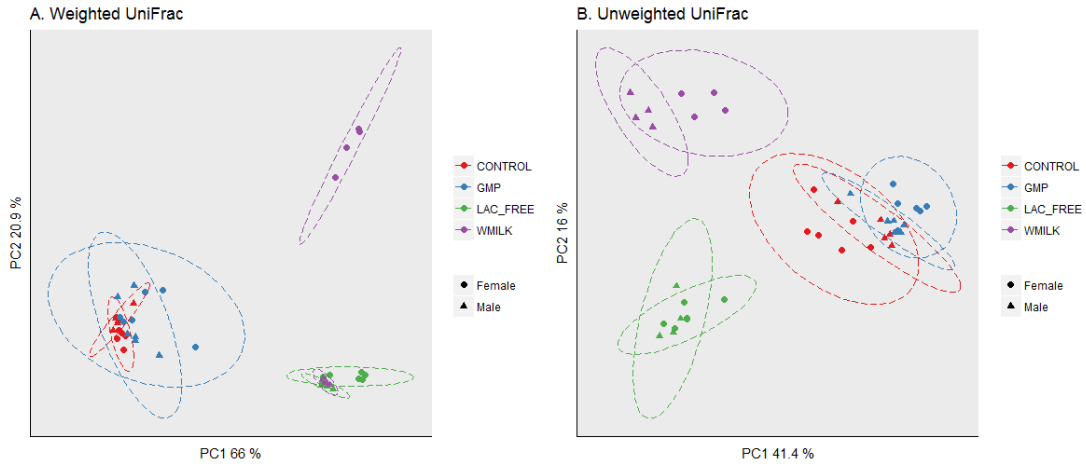
Faecal microbiota compositional profile of mice fed lactose-free milk supplemented diet and humanised with longstay type microbiota

At the end of the trial (T5) the faecal microbiota from mice humanised with LS microbiota and fed milk-supplemented diets clustered closer compared to the microbiota from mice fed GMP or control diet as shown in the PCoA in **Figures 18 A** and **B**. Compared to the other diets, the lactose-free diet resulted in the highest Porphyromonadaceae abundance increase (29% average relative abundance) represented predominantly by *Parabacteroides merdae* and to a lesser extent by *Parabacteroides distasonis* (**Figures 19, 20; S Figure 3 B**). Lachnospiraceae (16% average relative abundance), Rikenellaceae and Bacteroidaceae (14% average relative abundance each) were the next most abundant families (**Figure 19**). The families Desulfovibrionaceae (*Desulfovibrio piger*), Peptostreptococcaceae (*C. ruminantium*), Ruminococcaceae (*F. plautii*) and Enterobacteriaceae (*Shigella* taxa) reached the highest abundance after the lac-free diet feeding compared to the other diets (**Figures 19**, **20**). Limited differences in the composition of the faecal microbiota between female and male mice, based on abundances of certain families such as the Bacteroidaceae (more abundant in male) and Porphyromonadaceae (more abundant in female) (**Table 8, S Table 7**) may explain the sub-clustering within the Weighted PCoA (**Figure 18 A**).

4282 

4283 **Figure 18 Principal coordinates analysis (PCoA) of the murine faecal**
4284 **microbiota at the end of the trial (T5).** The mice were humanised with long stay
4285 (**LS**) type faecal microbiota. **A**: Weighted UniFrac distances; **B**: Unweighted
4286 UniFrac distances. LAC_FREE: lactose-free milk supplemented diet, WMILK:
4287 whole milk supplemented diet, GMP: glycomacropeptide supplemented diet,
4288 CONTROL: soy protein based control diet. ."●" denotes female mouse microbiota;
4289 "▲" denotes male mouse microbiota.

Gender dependent enrichment of Lachnospiraceae and Erysipelotrichaceae due to whole milk supplemented diet in mice humanised with longstay type microbiota.

The Weighted UniFrac analysis of the faecal microbiota of mice fed the whole milk diet, resulted in two sub-clusters based on gender-dependent responses, (**Figures 18 A**). Different Lachnospiraceae taxa were responsive in the microbiota of female and male mice whereas the Erysipelotrichaceae represented by *A. stercorianis* increased only in the female mice microbiota (**Table 8**). The Bacteroidaceae that were among the most abundant families in both female and male mouse microbiota (19% average relative abundance in both) had gender associated differences in the responsive taxa (**S Table 7**, **Table 8**). Other dominant taxa with gender associated variations in the relative abundance belonged to the Porphyromonadaceae and Rikenellaceae (**Table 8**). The aggregated across gender mouse microbiota composition is shown in **Figures 19** and **20**. Compared to the other feeding regimes, Lachnospiraceae and Erysipelotrichaceae were significantly enriched upon the whole milk diet and compared to the other feeding regimes (29% and 19% average relative abundance respectively) (**S Figure 3 B**).

Effect of GMP and control diet on the longstay type microbiota

The Bacteroidaceae was the second most abundant family (25% average relative abundance) in the microbiota of mice fed GMP diet following the Verrucomicrobiaceae, with *B. dorei* and *B. thetaiotaomicron* as the main representatives of the Bacteroidaceae family (**Figures 19, 20**). Compared to the other feeding regimes, GMP diet sustained the highest Bacteroidaceae and

Acidaminococcaceae (represented by *P. faecium*) relative abundances in the faecal microbiota of mice humanised with LS microbiota (**Figures 19, 20; S Figure 3 B**).

Similar to aforementioned feeding regimes, the Verrucomicrobia were overrepresented in the faecal microbiota of mice fed the control diet and humanised with LS microbiota (**Figure 19**). Compared to the other diets, the control diet resulted in enrichment of the microbiota in the Rikenellaceae family (22% average relative abundance) (**S Figure 3 B**) and *Alistipes putredinis* was the prevalent representative taxon (**Figure 20**). The Ruminococcaceae (represented by *F. plautii* and other unclassified taxa) had a relatively high abundance comparable to that observed under the lactose-free feeding regime whereas the families Lachnospiraceae, Bacteroidaceae and Porphyromonadaceae had the lowest abundance compared to the other diets (**Figure 19**).

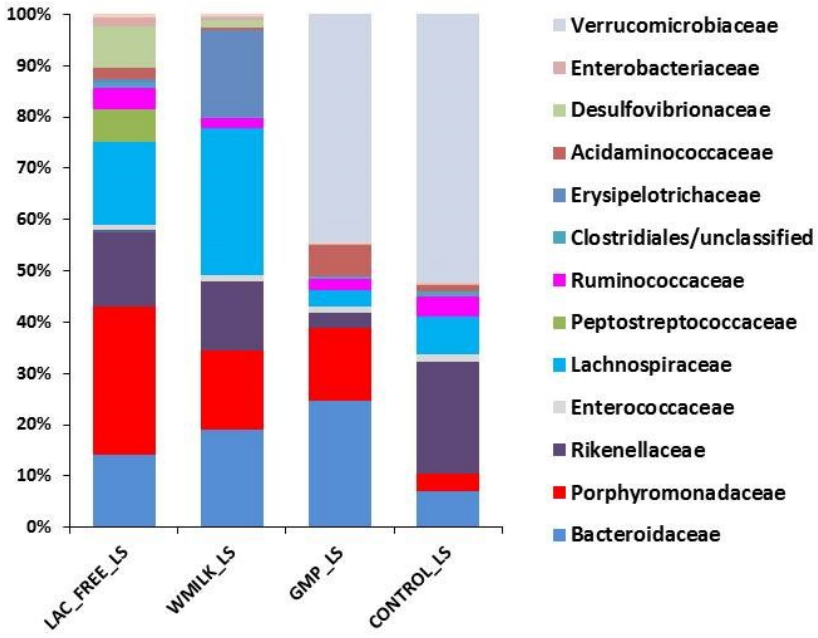


Figure 19 Composition of the murine faecal microbiota at family level at the end of the trial (T5) in mice humanised with longstay (LS) type human faecal microbiota. LAC_FREE: lactose-free milk, WMILK: whole milk, GMP: glycomacropeptide, CONTROL: soy-protein based control diet. Only families present at ≥1 % relative abundance are shown.

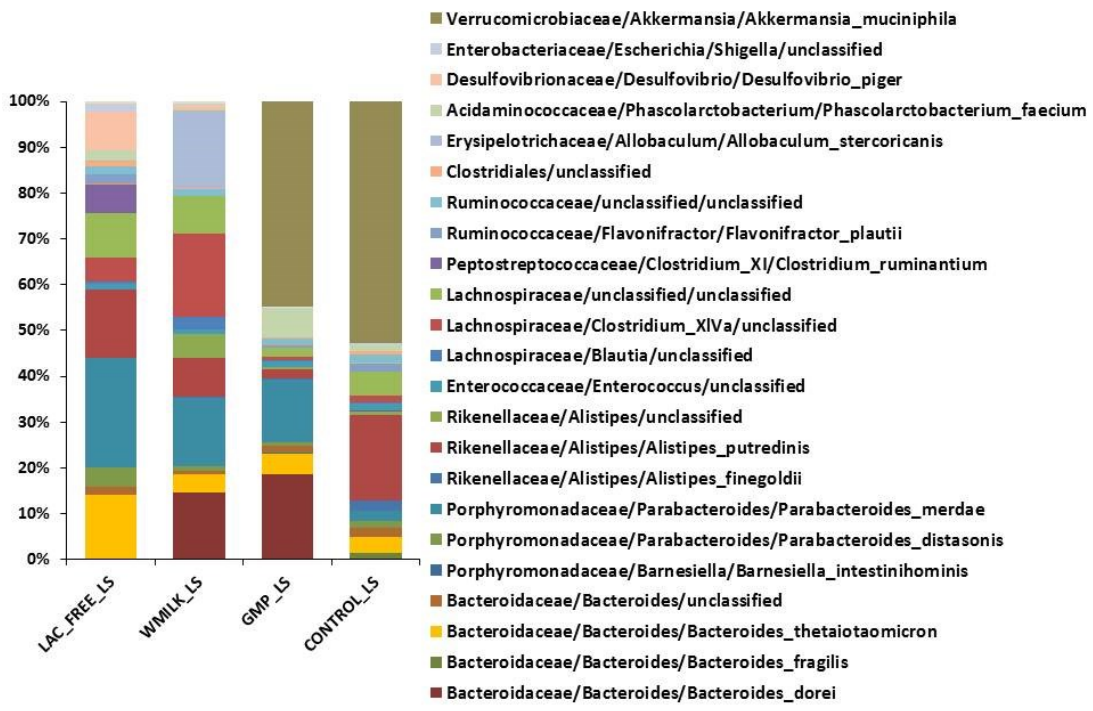


Figure 20 Composition of the murine faecal microbiota at family level at the end of the trial in mice humanised with longstay (LS) type human faecal microbiota. LAC_FREE: lactose-free milk, WMILK: whole milk, GMP: glycomacropeptide, CONTROL: soy-protein based control diet. Only families present at ≥1 % relative abundance are shown.

Table 8 Differences in the female and male murine microbiota composition associated with separation in the PCoA grouping by lactose-free milk and whole milk diets. Only species that were present at ≥1 % relative abundance are presented. The humanisation was performed with longstay (**LS**) microbiota.

	LAC_FREE FEMALE	LAC_FREE MALE	WMILK FEMALE	WMILK MALE
OTU classification	**% average relative abundance**			
Bacteroidaceae/Bacteroides/Bacteroides dorei	0.0	0.0	9.9	18.7
Bacteroidaceae/Bacteroides/Bacteroides thetaiotaomicron	8.6	25.0	7.8	0.002
Bacteroidaceae/Bacteroides/unclassified	0.90	3.5	1.4	0.003
Porphyromonadaceae/Parabacteroides/Parabacteroides distasonis	4.3	3.8	0.5	1.5
Porphyromonadaceae/Parabacteroides/Parabacteroides merdae	28.2	14.9	3.1	26.0
Rikenellaceae/Alistipes/Alistipes putredinis	13.5	15.3	5.1	11.8
Enterococcaceae/Enterococcus/unclassified	1.0	1.4	0.002	2.2
Lachnospiraceae/Blautia/unclassified	0.6	0.2	4.6	0.9
Lachnospiraceae/Clostridium XlVa/unclassified	5.8	2.4	29.7	5.6
Lachnospiraceae/unclassified/unclassified	11.6	7.9	0.2	15.4
Peptostreptococcaceae/Clostridium_XI/Clostridium ruminantium	6.5	4.6	0.001	0.0
Ruminococcaceae/Flavonifractor/Flavonifractor plautii	1.7	2.9	0.1	0.10
Ruminococcaceae/unclassified/unclassified	2.1	2.0	0.1	3.0
Clostridiales/unclassified/unclassified/unclassified	1.2	0.8	0.2	0.4
Erysipelotrichaceae/Allobaculum/Allobaculum stercoricanis	0.001	0.001	32.3	0.01
Acidaminococcaceae/Phascolarctobacterium/ Phascolarctobacterium faecium	2.2	1.8	1.1	0.002
Desulfovibrionaceae/Desulfovibrio/Desulfovibrio piger	8.0	8.0	1.4	0.0
Desulfovibrionaceae/unclassified/unclassified	0.1	0.01	0.0	1.2
Enterobacteriales/Enterobacteriaceae/Escherichia/ Shigella/unclassified	1.1	2.7	0.5	0.001

Enrichment of the microbiota in Verrucomicrobiaceae in some dietary groups

In the faecal microbiota of some groups humanised with either microbiota type, an enrichment (≥ 40% average relative abundance) in the Verrucomicrobiaceae family represented by *A. muciniphila* was observed at the end of the trial (T5) (**Figures 17**, **20**). Alpha diversity scores may have been biased due to the *Akkermansia* bloom observed at (T5) and for this reason the microbiota composition and alpha diversity

indices were analysed at an earlier time point, i.e. in the middle of the dietary intervention (T4) (**S Figure 4**, **S Tables 8, 9**). The Verrucomicrobiaceae bloom was detected at time point T4 for the mice humanised with COM microbiota but not for the mice humanised with LS type microbiota (**S Figure 4**). At T4, the lactose-free diet sustained significantly higher alpha diversity in the murine microbiota of COM type humanised mice compared to the other feeding regimes; however, the Verrucomicrobiaceae bloom may have contributed to the alpha diversity observed for control feeding (**S Table 8**). For the LS type humanised mice at T4, both milk supplemented diets sustained significantly higher alpha diversity indices compared to both GMP and control diet (**S Table 8**).

3.5 Discussion

Prebiotic effect of the lactose-free milk supplemented diet on the gut microbiota of mice humanised with faecal microbiota from older donors

In mice colonised with either human microbiota type the lactose-free diet sustained significantly higher faecal microbiota diversity compared to the whole milk or GMP diets, performing as efficiently as soy protein supplemented diet. Some animal and human studies indicate to the prebiotic potential of soy and its products (reviewed in Huang *et al*., 2016). Decreased gut microbiota phylogenetic diversity is indication biomarker of ageing and frailty, and dietary supplements contributing in sustaining diversity may improve the ageing process (Claesson *et al*., 2012; Jackson *et al*., 2016). The efficiency of the lactose-free milk diet in retaining microbiota diversity can be attributed partly to the BMOs available for microbiota fermentation. Bovine milk oligosaccharides have attracted scientific interest as potential prebiotics due to

their structural complexity and similarity to HMOs (Zivkovic and Barile, 2011; Smilowitz *et al*., 2014).

The Ruminococcaceae enrichment of the microbiota associated with the lactose-free diet associated observed mostly in COM type humanised animals could indicate that such a dietary supplementation might contribute to colonic health and healthy ageing as reported before (Kong *et al*., 2016; Mottawea *et al*., 2016). In the current study the dominant Ruminococcaceae taxon was *Anaerotruncus*. Others have reported probiotic-associated *Anaerotruncus* enrichment of dysbiotic murine microbiota after Abx treatment (Grazul *et al*., 2016). Other dominant Ruminococcaceae taxa reported here i.e., *Oscillibacter* and *Flavonifractor*, have been associated with resistant starch degradation and identified in the gut microbiota of lean individuals (Walker *et al*., 2011; Kasai *et al*., 2015). However, caution is needed before extrapolating these findings to human dietary interventions because some studies report enrichment of *Oscillibacter* in cancer associated microbiota and *Anaerotruncus* enrichment in the microbiota of frail elderly (Jeffery *et al*., 2016; Flemer *et al*., 2017). In the LS humanised mice the Ruminococcaceae reached the highest relative abundance after lactose-free (and control) diet but not as high as in COM type humanised mice, potentially due to low starting abundance of this family in the LS microbiota. Importantly, in LS humanised mice, lac-free diet resulted in increased *D. piger* relative abundance; a taxon negatively correlated to health in elderly people (Jeffery *et al*., 2016). The balance of these effects needs to be measured and considered in human studies.

Prebiotic potential of whole milk

The whole milk supplemented diet was less efficient than the lactose-free diet in sustaining microbiota diversity. However, the comparative increase in the relative abundance of certain taxa after wmilk diet warrants further investigation. *Ruminococcus bromii* (Ruminococcaceae) was detected at high abundance exclusively in (male) mice humanised with COM type microbiota fed whole milk diet. *Ruminococcus bromii* is a dominant gut microbiota taxon highly responsive to resistant starch, essential in nutrient degradation and as such it can be a prebiotic target leading to substrate release in the colon and increase in cross-feeding mediated by SCFA production (Ze *et al*., 2012; Scott *et al*., 2015). The (gender dependent) Erysipelotrichaceae/*A. stercorianis* enrichment of the faecal microbiota of whole milk fed mice may be a positive effect on the microbiota. In murine trials, high-fat diet-associated dysbiosis and impaired gut barrier function were restored after BMOs supplementation correlating to *Allobaculum* (and Ruminococcaceae) enrichment of the microbiota (Boudry *et al*., 2017; Hamilton *et al*., 2017). Inulin supplementation had similar effect on *Allobaculum* taxa associated with improved vascular function (Catry *et al*. 2018). However, careful extrapolation to human diet is needed due to *Allobaculum* association with thrombosis risk through TMAO metabolism and to other erysipelotrichi associations with the obesity phenotype in murine models (Woting *et al*., 2014; Zhu *et al*., 2016). Importantly the whole milk diet, similarly to the lactose-free diet, sustained the highest Lachnospiraceae abundance in the murine microbiota compared to the control and GMP diets, and especially in LS humanised mice the whole milk diet resulted in a favourable ratio of Lachnospiraceae and Bacteroidaceae. In aged humans with frailty indications, Bacteroidaceae were found to dominate in abundance over Lachnospiraceae (Jeffery *et al*., 2016). Interestingly, the increased relative ratio of Lachnospiraceae to Bacteroidaceae may explain the

fact that milk-supplemented diets resulted in the highest weight gain from baseline (**Figure 11**) (Turnbaugh *et al*., 2006; Jumpertz *et al.,* 2011).

GMP was not associated with prebiotic activity

The Bacteroidetes (Bacteroidaceae, Bacteroidales unclassified and Porphyromonadaceae), Enterobacteriaceae and Desulfovibrionaceae were responsive to GMP and the milk diets depending on humanisation type (**S Figure 3**). In this study we did not observe *in vivo* beneficial effects of the GMP supplementation on the gut microbiota in contrast to previous studies in a conventional mouse model (Sawin *et al*., 2015). In our study increased Desulfovibrionaceae/*B. wadsworthia* abundance due to GMP supplementation in COM humanised mice was observed, contrary to previous studies reporting decreased Desulfovibrionaceae/*D. piger* relative abundance associated with 20% GMP-enriched diet (Sawin *et al*., 2015). Intake of high-fat diet (milk-derived fat) resulted in *B. wadsworthia* associated dysbiosis and inflammation in a conventional mouse model (Devkota *et al*., 2012). Colonic health is promoted by reduced *Bilophila* relative abundances in human gut microbiota (Vandeputte *et al*., 2017). The sialic acid content of the GMP may have contributed to the comparative increase in Enterobacteriaceae/*Escherichia*/*Shigella* taxa relative abundance in COM type humanised mice microbiota (Huang *et al*., 2015). A similar response was not observed in LS humanised mice; the Enterobacteriaceae were more responsive to the lactose-free milk diet. In the microbiota of LS colonised mice the Bacteroidaceae were highly responsive to GMP-supplemented diet (and the other two dairy diets). Increase in *Bacteroides* population due to GMP-supplementation has been reported in mouse models (Sawin *et al*., 2015; Jimenez *et al*., 2017). Interestingly, different Bacteroidaceae taxa have

been associated with sialidase activity with or without sialic acid utilisation ability (Huang *et al.,* 2015; reviewed in Juge *et al*., 2016). Charbonneau *et al*. (2016) reported growth improvement in a gnotobiotic mouse and piglet model through cross-feeding between *Bacteroides* taxa that could degrade sialic acid-containing carbohydrates from sialylated BMO and secondary utilisers of sialic acid-containing compounds like *E. coli*.

In Chapter 2, I reported the increased relative abundance of Lachnospiraceae taxa like *Coprococcus* and *Roseburia* due to GMP *in vitro* supplementation; in this *in vivo* model we did not observe similar microbiota responsiveness. As discussed in the following section (and in details in Chapter 1.2.4 of this thesis), the mouse gut may favour the colonisation of xenomicrobiota Bacteroidetes taxa and may be refractory to Lachnospiraceae and other Firmicutes taxa colonisation. This could partly explain why, in this *in vivo* model, Lachnospiraceae taxa were not observed to be major responders to GMP supplementation.**Establishment of the human faecal microbiota in the murine GIT tract**

Although the faecal microbiota across groups at the end of the trial strongly separated from that at baseline grouping by humanisation type and diet (**S Figures 2, 3**), it is considered that the murine host played a role in re-shaping the xenomicrobiota. For example, at the end of the trial, the Bacteroidetes and especially Porphyromonadaceae enrichment across all dietary groups and the compositional profile of the Lachnospiraceae can be viewed as the result of selective pressure applied by the murine host to reshape the relative abundances of shared humans xenomicrobiota taxa to best fit murine host physiology and immunity (Nguyen et al., 2015; Messer *et al*., 2016). In this study, similarly to previous observations, we

observed gender dependent differential responses to diet partly attributed to hormonal effects (Org *et al*., 2016; Wang *et al*., 2016). The *Akkermansia* bloom could be attributed to the HydroGel treatment that interrupted the Abx treatment in some dietary groups, to coprophagy and to cross-contamination between cages. Interestingly, Dubourg *et al*. (2012) reported increased *A*. *muciniphila* colonisation in gut microbiota after broad-spectrum Abx treatment.

3.6 Conclusions

Milk is a widely accessible dietary product, it combines an array of valuable nutrients and many recent human cohort studies have demonstrated the health benefits of milk consumption (Haug *et al*., 2007; Visioli and Strata, 2014; Guo *et al*., 2017). Importantly, BMOs may be an efficient prebiotic substrate similarly to HMOs (Zivkovic and Barile, 2011; Smilowitz *et al*., 2014). We have discussed the gut microbiota modulatory potential of (lactose free and whole) milk in a murine model. Lactose free milk was found a better prebiotic candidate compared to whole milk as it retained higher microbiota diversity. However, whole milk diet affected the abundance of significant health-relevant microbiota taxa. When translating the findings of the current study to humans, the fact that mice may not fully tolerate lactose (van de Heijning *et al*., 2015), and that the lactose fermentation in wmilk diet fed mice may have prevented BMOs utilisation by the gut microbiota, must be taken into consideration. If the two milk types, i.e., whole milk and lactose-free milk, have a differential effect on the gut microbiota of lactase persistent subjects remains to be clarified in a clinical trial.

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3.9 References

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3.10 Supplementary Chapter 3

S Table 1 Mouse treatment groups tested by diet, gender and the human microbiota type. The number of mice at the end of the trial per group is indicated. The experimental arms of groups A, B, C and D were performed simultaneously followed by the experimental arms of groups E, F, G and H. The animals that received HydroGel treatment during Abx period, is indicated.

	Community type human microbiota (COM)		**Longstay type human microbiota (LS)**	
	female	**male**	**female**	**male**
Lactose free milk	A (n=5)	A (n=5)	B (n=6)	B (n=6)
Whole milk	C (n=6)	C (n=6)	D (n=4)	D (n=5)
GMP	E (n=6)#	E (n=6)#	G (n=6)	G (n=6)
Control	H (n=6)###	H (n=6)	F (n=6)##	F (n=5)###

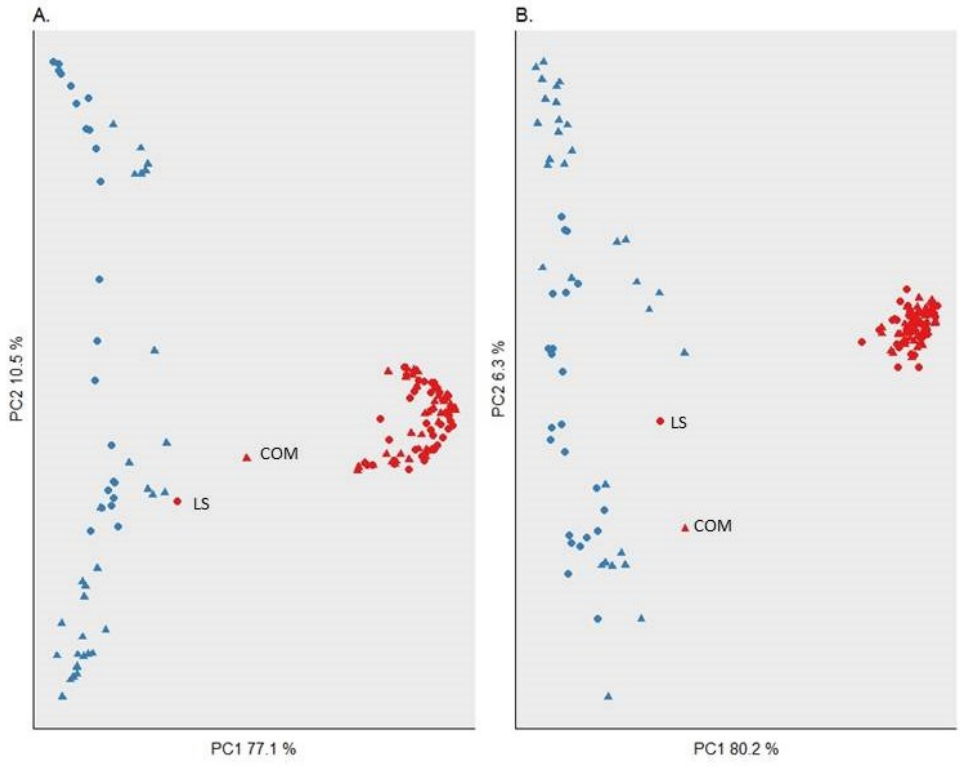
"#","##","###" three, six and nine days, respectively, of HydroGel intake during Abx treatment

S Table 2 Composition of the baseline murine and the human faecal microbiota. EM297; longstay type faecal microbiota (LS); EM425 community type faecal microbiota (COM). Only species present at ≥1 % relative abundance in at least one group are presented.

	MURINE	EM297 LS	EM425 COM
OTU classification	**% average relative abundance**		
Bifidobacteriaceae/Bifidobacterium/unclassified	0.0	1.1	0.0
Bacteroidaceae/Bacteroides/Bacteroides acidifaciens	1.5	0.01	0.7
Bacteroidaceae/Bacteroides/Bacteroides dorei	0.2	9.5	0.0
Bacteroidaceae/Bacteroides/Bacteroides fragilis	0.0001	15.4	1.4
Bacteroidaceae/Bacteroides/unclassified	0.1	8.0	0.6
Porphyromonadaceae/Barnesiella/Barnesiella intestinihominis	7.9	0.7	2.6
Porphyromonadaceae/Barnesiella/unclassified	1.1	0.02	0.2
Porphyromonadaceae/Odoribacter/Odoribacter splanchnicus	0.4	1.7	0.3
Porphyromonadaceae/Parabacteroides/Parabacteroides merdae	0.2	3.0	1.3
Porphyromonadaceae/unclassified/unclassified	34.6	0.02	0.03
Prevotellaceae/unclassified/unclassified	4.1	0.0	0.0
Rikenellaceae/Alistipes/Alistipes massiliensis	3.2	0.0	0.0
Rikenellaceae/Alistipes/Alistipes putredinis	0.04	0.7	1.3
Rikenellaceae/Alistipes/unclassified	0.8	2.5	0.8
Deferribacteraceae/Mucispirillum/Mucispirillum schaedleri	1.1	0.0	0.0
Methanobacteriaceae/Methanobrevibacter/ Methanobrevibacter smithii	0.0	1.6	0.3
Lactobacillaceae/Lactobacillus/Lactobacillus reuteri	0.8	0.04	0.0
Lactobacillaceae/Lactobacillus/unclassified	1.9	0.3	0.0
Clostridiaceae_1/Clostridium sensu stricto/Clostridium disporicum	0.0	0.0	1.5
Lachnospiraceae/Clostridium XlVa/unclassified	1.7	2.6	0.04
Lachnospiraceae/Ruminococcus2/Ruminococcus torques	0.003	2.2	0.1
Lachnospiraceae/unclassified/unclassified	24.6	0.8	18.3
Peptostreptococcaceae/Clostridium_XI/Clostridium ruminantium	0.0001	0.0	1.5
Ruminococcaceae/Clostridium IV/Clostridium leptum	0.02	3.9	0.004
Ruminococcaceae/unclassified/unclassified	1.9	4.2	3.8
Clostridiales/unclassified/unclassified/unclassified	5.2	0.8	10.2
Acidaminococcaceae/Succiniclasticum/Succiniclasticum ruminis	0.0	0.0	1.3
Firmicutes/unclassified/unclassified/unclassified/ unclassified/unclassified	0.6	0.01	4.7
Pseudomonadaceae/Pseudomonas/Pseudomonas aeruginosa	0.0001	4.2	0.003
Synergistaceae/Cloacibacillus/Cloacibacillus evryensis	0.0001	21.3	0.0
Anaeroplasmataceae/Anaeroplasma/unclassified	0.004	0.0	1.7
unclassified/unclassified/unclassified/unclassified/ unclassified/unclassified	1.1	0.5	2.5
Verrucomicrobiaceae/Akkermansia/Akkermansia muciniphila	0.8	0.0	0.0

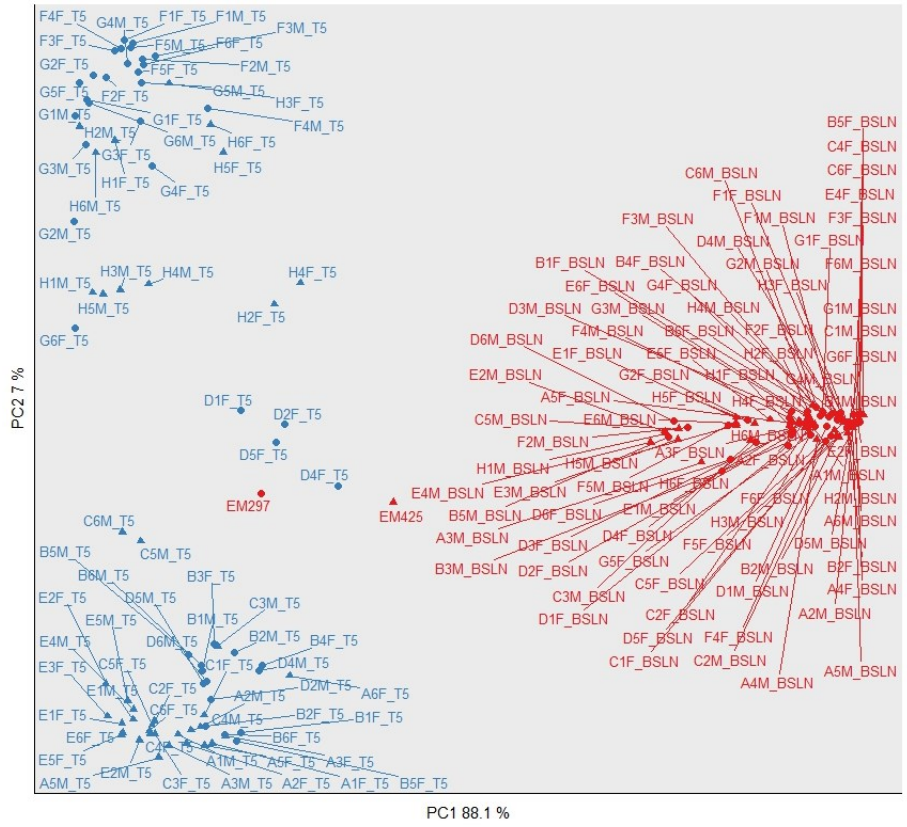
S Table 3 Alpha diversity indices of the faecal microbiotas of the human donors and the murine baseline faecal microbiota. EM297: longstay type faecal microbiota (LS); EM425: community type faecal microbiota (COM); murine: aggregated faecal microbiota across all mice at baseline.

Alpha Diversity Index	MURINE	EM297 LS	EM425 COM
Shannon	6.0	4.0	6.0
Simpson	1.0	1.0	1.0
PD Whole tree	15.0	9.0	17.0
Observed species	207.0	73.0	169.0
Chao 1	346.0	104.0	264.0

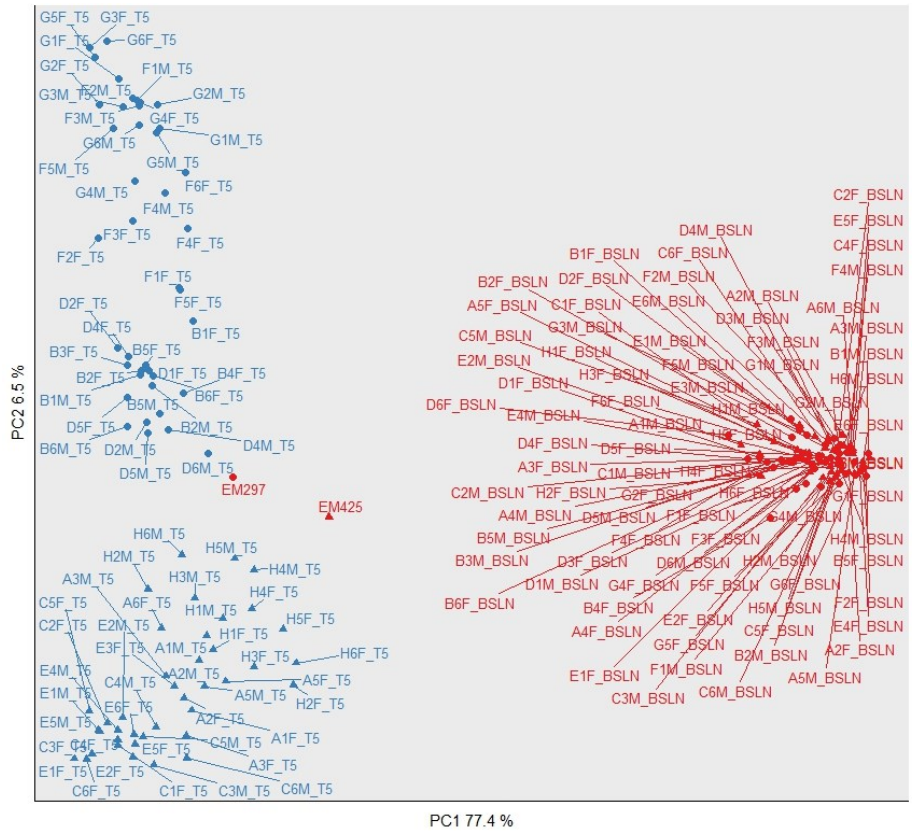


S Figure 1 Principal coordinates analysis (PCoA) of the UniFrac distances one week after "humanisation" (T3). A. Weighted UniFrac distances; **B**. Unweighted UniFrac distances; "▲" community (COM) type faecal microbiota; "●" longstay (LS) type faecal microbiota; **red**: baseline murine faecal microbiota; **blue**: T3 time point. The inocula of human faecal microbiota are denoted as COM and LS.

A.



B.



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S Figure 2 Principal coordinates analysis (PCoA) of the UniFrac distances at end of the trial (T5). **A**. Weighted UniFrac distances; **B**. Unweighted UniFrac distances; "▲" community (COM) type faecal microbiota; "●" longstay (LS) type faecal microbiota; **red**: baseline murine faecal microbiota; **blue**: T5 time point. The inocula of human faecal microbiota are denoted as COM and LS.

S Table 4 Alpha diversity statistical analysis for community (COM) type microbiota at the end of the trial (T5) LAC_FREE: lactose free diet; WMILK: whole milk diet; GMP: GMP diet; CONTROL: control diet.

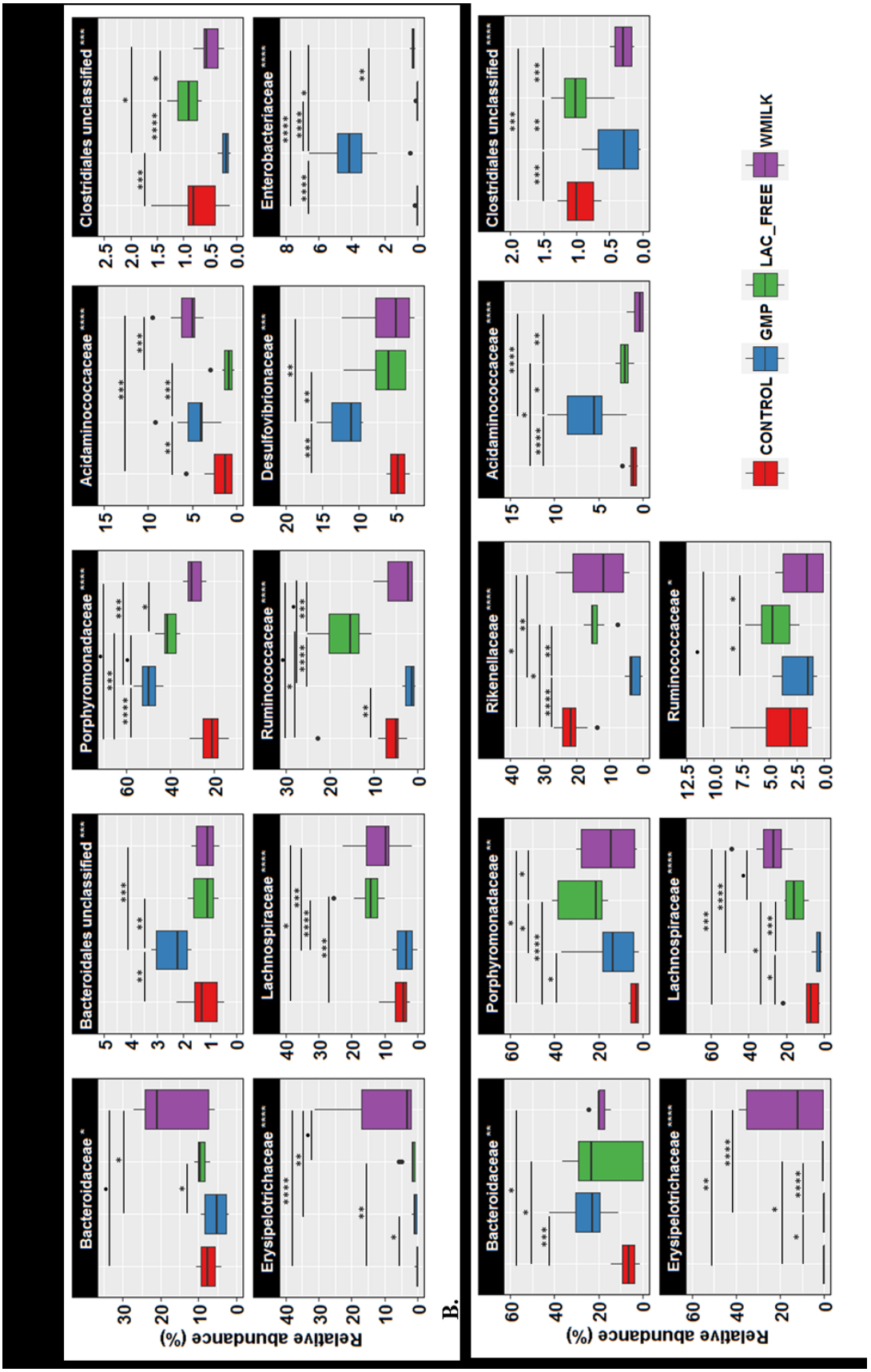
		Dunn's Post Hoc test p_{adj} values					
Diversity indices	**Kruskal-Wallis P_{adj}-value**	**CONTROL VS GMP**	**CONTROL VS LAC-FREE**	**GMP VS LAC-FREE**	**CONTROL VS WMILK**	**GMP VS WMILK**	**LAC-FREE VS WMILK**
Shannon	3.30E-05	0.0004	0.1	1.17E-05	0.2	0.01	0.03
Simpson	0.0002	0.0004	0.4	0.001	0.4	0.0003	0.4
PD	0.0001	0.0002	0.4	0.0003	0.02	0.06	0.02
Observed species	1.41E-06	1.20E-05	0.3	1.40E-05	0.004	0.06	0.003
Chao1	0.001	0.002	0.4	0.004	0.02	0.23	0.02

S Table 5 Alpha diversity statistical analysis for longstay (LS) type microbiota at the end of the trial (T5) LAC_FREE: lactose free diet; WMILK: whole milk diet; GMP: GMP diet; CONTROL: control diet.

		Dunn's Post Hoc test p_{adj} values					
Diversity indices	**Kruskal-Wallis P_{adj}-value**	**CONTROL VS GMP**	**CONTROL VS LAC-FREE**	**GMP VS LAC-FREE**	**CONTROL VS WMILK**	**GMP VS WMILK**	**LAC-FREE VS WMILK**
Shannon	3.83E-06	0.01	0.007	1.20E-06	0.1	0.2	0.0003
Simpson	0.0002	0.30	0.001	7.73E-05	0.4	0.3	0.003
PD	1.85E-05	0.004	0.1	5.01E-06	0.1	0.1	0.004
Observed species	8.68E-06	0.001	0.3	0.0004	0.0004	0.2	0.0002
Chao1	0.05	0.09	0.4	0.09	0.05	0.3	0.066

S Table 6 Differences in the female and male murine microbiota composition associated with separation in the PCoA grouping by whole milk and control diet. Only families that were present at ≥1 % relative abundance in at least one group are presented. WMILK: whole milk diet; CONROL: control diet; community (COM) type microbiota.

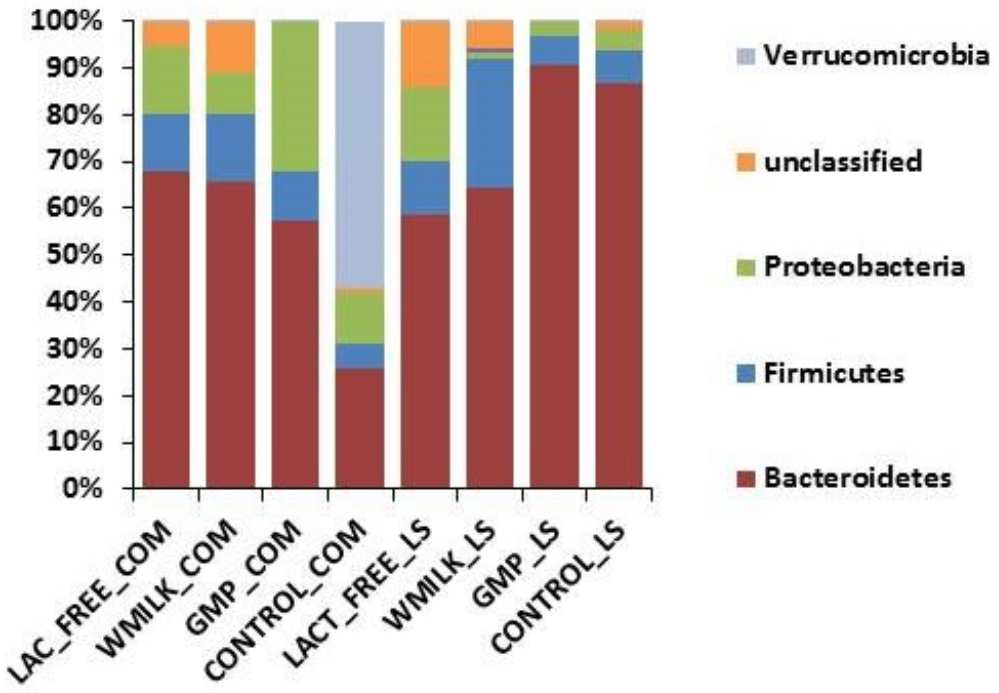
	WMILK FEMALE	WMILK MALE	CONTROL FEMALE	CONTROL MALE
OTU assignment	**% average relative abundance**			
Bacteroidaceae	23.6	6.6	7.3	7.7
Porphyromonadaceae	30.3	28.0	25.0	17.9
Rikenellaceae	6.7	9.9	0.8	18.1
Bacteroidales/unclassified	1.3	1.0	0.8	1.7
Lachnospiraceae	10.9	12.8	5.4	5.4
Peptostreptococcaceae	2.7	0.001	0.0	0.0
Ruminococcaceae	1.7	7.7	9.2	4.6
Clostridiales/unclassified	0.6	0.4	0.5	1.1
Erysipelotrichaceae	2.3	23.1	0.6	0.004
Acidaminococcaceae	5.7	5.5	3.0	0.6
Desulfovibrionaceae	7.7	3.0	4.5	5.0
unclassified	5.3	1.5	0.9	0.2
Verrucomicrobiaceae	0.002	0.0	40.6	37.2


B.
Relative abundance (%)
Bacteroidaceae *
Bacteroidales unclassified ***
Porphyromonadaceae ****
Acidaminococcaceae ****
Clostridiales unclassified ***
Erysipelotrichaceae ****
Lachnospiraceae ****
Ruminococcaceae ****
Desulfovibrionaceae ***
Enterobacteriaceae ****
Bacteroidaceae **
Porphyromonadaceae **
Rikenellaceae ****
Acidaminococcaceae ****
Clostridiales unclassified ****
Erysipelotrichaceae ****
Lachnospiraceae ****
Ruminococcaceae *
CONTROL
GMP
LAC_FREE
WMILK

S Figure 3 Differentially abundant taxa at family level at the end of the trial (T5) for aggregated female and male mouse faecal microbiota. A: community (COM) type colonisation; B: longstay (LS) type colonisation. Asterisks next to taxa denote the result of Kruskal-Wallis comparison; markings above boxplots denote the Dunn's post hoc result. Significant values: "●" padj<0.1; "*" padj<0.05; "**" padj<0.01; "***" padj<0.001; "****" padj<0.0001. COM type humanisation:

S Table 7 Differences in the female and male murine microbiota composition associated with separation in the PCoA grouping by the lac-free and whole milk diet. Only families that were present at ≥1 % relative abundance in at least one group are presented. LAC_FREE: lactose free diet; WMILK: whole milk diet; longstay (LS) type microbiota.

	LAC-FREE FEMALE	LAC-FREE MALE	WMILK FEMALE	WMILK MALE
	% average relative abundance			
Bacteroidaceae	9.5	28.5	19.1	18.7
Porphyromonadaceae	32.6	19.0	3.6	27.4
Rikenellaceae	13.5	15.4	5.4	21.6
Enterococcaceae	1.0	1.4	0.002	2.2
Lachnospiraceae	18.5	10.8	34.6	22.8
Peptostreptococcaceae	6.5	4.6	0.001	0.0
Ruminococcaceae	4.2	5.2	0.1	3.7
Clostridiales/unclassified	1.2	0.8	0.2	0.4
Erysipelotrichaceae	0.8	0.6	33.1	0.6
Acidaminococcaceae	2.2	1.8	1.1	0.002
Desulfovibrionaceae	8.1	8.0	1.4	1.2
Enterobacteriaceae	1.1	2.8	0.6	0.7



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4778 **S Figure 4 Composition of the murine faecal microbiota at phylum level at the**
4779 **mid part of the dietary intervention (T4).** COM: community type microbiota, LS:
4780 longstay type microbiota. LAC_FREE: lactose free milk, WMILK: whole milk,
4781 GMP: glycomacropeptide, CONTROL: soy protein based control diet. Only phyla
4782 present at ≥1 % relative abundance are presented.

S Table 8 Alpha diversity analysis for community (COM) type microbiota at the mid part of the trial (T4) LAC_FREE: lactose free diet; WMILK: whole milk diet; GMP: GMP diet; CONTROL: control diet.

		Dunn's Post Hoc test p_{adj} values					
Index	**Kruskal-Wallis P_{adj}-value**	**CONTROL VS GMP**	**CONTROL VS LAC-FREE**	**GMP VS LAC-FREE**	**CONTROL VS WMILK**	**GMP VS WMILK**	**LAC-FREE VS WMILK**
Shannon	1E-05	0.3	8E-05	7E-06	0.1	0.02	0.01
Simpson	1E-04	0.3	6E-05	0.0003	0.2	0.4	0.001
Observed species	0.0002	0.01	0.04	4E-05	0.4	0.01	0.05
PD whole tree	0.0002	0.003	0.21	0.0003	0.3	0.001	0.3
Chao1	0.001	0.02	0.05	0.0002	0.3	0.01	0.1

Alpha diversity average values				
Index	**CONTROL**	**GMP**	**LAC-FREE**	**WMILK**
Shannon	3	3	4	3
Simpson	1	1	1	1
Observed species	46	28	57	46
PD whole tree	5	3	6	6
Chao1	66	33	71	57

S Table 9 Alpha diversity analysis for longstay (LS) type microbiota at the mid part of the trial (T4) LAC-FREE: lactose free diet; WMILK: whole milk diet; GMP: GMP diet; CONTROL: control diet.

		Dunn's Post Hoc test p_{adj} values					
Index	**Kruskal-Wallis P_{adj}-value**	**CONTROL VS GMP**	**CONTROL VS LAC-FREE**	**GMP VS LAC-FREE**	**CONTROL VS WMILK**	**GMP VS WMILK**	**LAC-FREE VS WMILK**
Shannon	1E-06	0.4	0.0001	0.00004	0.0008	0.0002	0.4
Simpson	2E-06	0.5	0.0001	0.0001	0.0003	0.0002	0.5
PD whole tree	1E-06	0.2	0.0007	1E-05	0.002	6E-05	0.4
Observed species	1E-06	0.4	0.0001	4E-05	0.001	0.0002	0.4
Chao1	1E-06	0.4	0.0004	9E-05	0.0003	5E-05	0.4

	Alpha diversity average values			
Index	**CONTROL**	**GMP**	**LAC-FREE**	**WMILK**
Shannon	2	2	4	4
Simpson	1	1	1	1
PD whole tree	2	1	3	3
Observed species	14	12	31	31
Chao1	16	14	39	42

Chapter 4

Effect of blueberry on the composition and metabolism activity of gut microbiota and artificial microbiota community from the elderly

4.1 Abstract

We tested the modulatory effect of freeze-dried blueberry powder on the human gut microbiota of older subjects using an *in vitro* batch fermentation system. The blueberry powder was rich in polyphenols and fibre. In older people, lower gut microbiota diversity is associated with poor health and mortality. Therefore, two types of faecal microbiota were used, i.e. low diversity longstay type (LS) microbiota from older subjects residing in long-term care units, and higher diversity community type (COM) microbiota from community-dwelling subjects. An artificial gut microbiota community (MCC100) of 100 strains designed to emulate the compositional profile of the gut microbiota of a healthy adult, was also used. The fermentation medium was supplemented with either blueberry powder or prebiotic mix alone, or a combination of the two supplementation regimes. The microbiota was analysed at baseline and at time points 16 h and 24 h of fermentation by Illumina MiSeq amplicon sequencing of the 16S rRNA gene. The differential microbiota responsiveness to the blueberry supplementation was associated with the baseline microbiota composition. Blueberry supplementation resulted in increased Lactobacillales/*Streptococcus* relative abundance most likely due to the high sugar content of the supplement. The blueberry supplementation increased the relative abundance of Bifidobacteriales/*Bifidobacterium* when the taxon was abundant at baseline. The increase in the relative abundance of the Proteobacteria across all microbiotas tested could be the result of simple sugar fermentation. In the lower complexity MCC100 artificial microbiota the combined supplementation resulted in comparatively increased alpha diversity; a similar result was not observed in the tested faecal microbiotas. The combined supplementation resulted in increased short chain fatty acid production in MCC100 and the faecal microbiota. The high sugar

content of the whole blueberry powder had a stong effect on the observed microbiota responses to blueberry supplementation. Follow-up *in vitro* trials with isolated phenolic compounds such as anthocyanins will elucidate the role of the gut microbiota in the bioconversion of blueberry polyphenol extracts.

4.2 Introduction

The gut microbiota is a recognised component of human health and it is shaped by host genetics, environment, life-style and diet (Sonneburg and Backhed, 2016; Messer *et al*., 2017). Based mostly on cohorts representing western populations and life-style, and despite significant inter-individual variations in gut microbiota composition, the existing data has enabled a general description of the healthy adult gut microbiota (Marchesi *et al*., 2015). Various events throughout the lifespan such as excessive antibiotic use, changes in dietary habits, and infections may ultimately cause dysbiosis, that is, perturbations in the composition and reduction in the phylogenetic diversity of the gut microbiota that is associated with disease (Petersen and Round, 2014).

Immuno-senescence, hospitalization and changes in dietary habits may collectively contribute to age-related microbiota alterations observed in the gut microbiota of older individuals and to the increase of the inflammatory status in the elderly, a known risk factor for mortality in humans and animal models (Jeffery *et al*., 2015; Theveranjan *et al*., 2017). In conditions where dysbiosis is a disease feature, manipulation of the gut microbiota can be a tool for prevention, improvement or even therapy (Salazar *et al*., 2014). Probiotics, faecal microbiota transplants (FMT), live bacteriotherapy, prebiotics and supplementation such as with polyphenols are potential mechanisms for gut microbiota modulation (Petrof *et al*., 2013; Marchesi *et al*., 2015).

Flavonoids are polyphenolic compounds that naturally occur in fruits, vegetables and other plant-derived dietary components and can be sub-divided in to seven sub-classes including flavones, and anthocyanins (reviewed in Panche *et al*., 2016).

Anthocyanins are present in plants as glycosylated anthocyanidins conjugated with sugars including glucose, galactose, arabinose, rhamnose and xylose (reviewed in Pojer *et al*., 2013). Dietary intake of these compounds has been associated with health benefits based on *in vitro* and *in vivo* experimental models and human studies (reviewed in Li *et al*., 2017). Ivey *et al*. (2017) reported that flavonoid-rich diet was associated with improved health and reduced mortality in a follow-up cohort study of young and middle-aged adults.

The reported flavonoid consumption in adult populations varies due to differences in the analytical methods used to assess the flavonoid content in food products, and because of widely varying dietary habits (reviewed in Chun *et al*., 2007; Chun *et al*., 2012; Pojer *et al*., 2013). Adults in the US, Europe and the UK have a daily consumption of flavonoids that ranges from 177 mg/d up to 428 mg/d and consumption of anthocyanidins that ranges from 4.2 mg/d up to 19 mg/d (Chun *et al*. 2007; Beking and Vieira 2011;Vogiatzoglou *et al*. 2015).

Unabsorbed phenolic compounds reach the colon where they may serve as substrates for faecal microbiota fermentation (reviewed in Selma *et al*. 2009). Several *in vitro* studies (Sánchez-Patán *et al*., 2012; Hidalgo *et al*., 2012; Cueva *et al*., 2013; Sánchez-Patán *et al*., 2015; Zhang *et al*., 2016) and *in vivo* studies (Del Bo *et al*., 2010; Lacombe *et al*., 2013; Fotschki *et al*., 2016; Collins *et al*., 2016) indicate towards the potential of polyphenols to modulate the gut microbiota. There is also scientific interest in the combined effect of dietary polyphenols and fibre on the gut microbiota and health (Edwards *et al*., 2017).

In this study, we applied 16S rRNA gene amplicon sequencing in order to describe the compositional changes that occurred in the faecal microbiota of older (>65 yrs)

individuals after 24 h of pH and temperature controlled *in vitro* fermentations supplemented with whole blueberry powder. Faecal samples collected from frail and healthy older people were used to differentiate the responsiveness of low and higher phylogenetic diversity faecal microbiota respectively to the selected substrates. To move beyond whole community analysis, identifying the functional properties of isolated gut bacteria will further enable preclinical studies towards targeted restoration of dysbiosis based on artificial bacterial consortia (Clavel *et al*., 2017). We therefore also tested an artificial bacterial consortium consisting of isolated faecal bacteria from healthy donors and representing the faecal microbiota of a healthy adult in the 24 h *in vitro* batch fermentation system for its fermentative potential on the blueberry powder.

4.3 Materials and Methods

Faecal Samples: Faecal samples were collected from two community-dwelling (COM) healthy older people (EM425 male 81 yrs, EM278 female 69 yrs) and two frail long-term care unit-residing (LS) older people (EM297 female 82 yrs, EM704 male 89 yrs). The local Clinical Research Ethics Committee approved all practices. The faecal samples were transferred to an anaerobic cabinet within an hour after passing. Several 10% w/v slurries were prepared from each homogenised faecal sample using sterile reduced phosphate buffer saline (PBS) with 20% v/v glycerol. All faecal slurries were aliquoted and stored frozen at -80°C.

Artificial gut microbiota MCC100: This artificial bacterial consortium consisted of 100 strains of strict anaerobic and facultative anaerobic bacteria isolated from faeces of healthy individuals and it was assembled based on the composition of the gut microbiota profile of healthy western adult (Perez *et al*., in preparation). The

bacterial consortium was premixed from individual purified cultures, concentrated and stored in -80°C.

Single stage chemostat: Batch fermentations were used in order to simulate colonic bacterial fermentation of the selected substrates (Hidalgo *et al*., 2012). The fermentation conditions were as described before with some variations (Ntemiri *et al*., 2017). The temperature and pH were kept stable at 37°C and 7.0 respectively. The duration of the fermentation was 24 h. The fermentation vessel was purged continuously with NO_2 and the working volume used was 400 ml. The basal medium was supplemented with 3 g/l of casein and antifoam A emulsion (1 ml/l) (Sigma Aldrich). The vessels were inoculated in triplicate at 1% w/v faecal sample; faecal samples were treated individually. Ten ml of thoroughly stirred fermentation liquid was retrieved from the fermenter vessel for analysis at time points 0 h and 16 h. This volume (10 ml) was selected in order not to reduce significantly the total volume of the fermentation. At time point 24 h, 50 ml faecal slurry was retrieved. The fermentation samples were immediately centrifuged and both supernatant and pellet were kept at -20°C for future analysis.

Fermentation Substrates: The basal medium was supplemented in three different ways: A: 1% w/v a mix of complex carbohydrates (MIX) consisting of xylan from corncob (0.6 g/l), pectin from apple (0.6 g/l), amylopectin (0.6 g/l), arabinogalactan (0.6 g/l), soluble starch (4.0 g/l), β-glucan (0.1 g/l), mucin type II (1.0 gr/l), glucose (2.0 g/l); B: 1% w/v MIX supplemented with 1.5 % w/v blueberry powder (BB.MIX); C:. 1.5 % blueberry powder (BB) alone. The blueberry powder was freeze-dried whole fruit powder, 50:50 tifblue:rubel blend; courtesy of the U.S. Highbush Blueberry Council, California) containing 21.4 g/100g of dietary fibre

(16.8 g insoluble fibre, 4.6 g soluble fibre). The BB dose was calculated to approach that of a high total flavonoid and anthocyanins daily dietary intake; approximately 200 mg per day and 70 mg per day respectively (Beking and Vieira, 2011) in an approximately 400 ml of simulated wet colon content (Sender *et al*., 2016). The BB powder contained 33 mg/g phenolics and 10.2 mg/g anthocyanins.

Bacterial DNA Extraction: Faecal pellets were weighted and resuspended in sterile PBS in order to provide approximately 200 mg of pellet required for the QIamp Fast DNA Stool (Qiagen) extraction kit protocol. The samples were homogenised mechanically in sterile tubes containing InhibitEX solution and zirconia glass beads of three sizes 0.1, 0.5 and 1.0 mm (Thistle Scientific, UK) using a Minibeadbeader (Biospec Products). Subsequent steps of gDNA extraction were performed as described before (Ntemiri *et al*., 2017).

Preparation of Illumina Library for 16S rRNA Amplicon Sequencing: The variable region V3/V4 of the 16S rRNA gene was targeted for amplicon sequencing using the Illumina MiSeq System (San Diego, California, USA). The universal 16S rRNA gene primers were forward primer for V3 region 5' TCGTCGGC AGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG 3' and reverse primer for V4 region 5' GTCTCGTGGGCTCGGAGATGTGTATAAG AGACAGGACTACHVGGGTATCTAATCC 3' (Klindworth *et al*. 2012). The amplicons were purified using the Agencourt AMPure XP-PCR Purification system (Beckman Coulter, Inc.). Dual index barcoding was performed to the purified products following the Illumina MiSeq System Protocol (Nextera XT V.2 Index Kits sets A and D, Illumina). The barcoded amplicons were purified (Agencourt AMPure XP-PCR Purification system; Beckman Coulter, Inc.) and quantified using the Qubit

dsDNA HS Assay Kit (Thermo Fischer Scientific, MA, U.S.A.). Each sample was pooled at the same concentration and sequenced in the Teagasc Next Generation Sequencing Facility (Fermoy, Ireland) on a MiSeq Illumina platform using 2x250 bp chemistry.

Analysis of 16S rRNA Amplicon Sequencing: Paired reads were joined using FLASH (Magoč *et al*., 2011) and demultiplexed in Qiime using the split_libraries.py script. The forward and reverse primers are removed and operational taxonomic unit (OTU) table was generated *de novo* with Usearch. Filtering of the reads based on the read length and the number of identical sequences was performed. Subsequently, chimera filtering was performed and mapping of all the reads against the representative reads generated by the filtering, based on 97% identity. The reads per sample were rarefied and the sequences were aligned using the Qiime PyNast tool (Caporaso *et al*., 2010). Alpha diversity indices i.e., Shannon, Simpson, PD whole tree, Chao1 and observed species, and beta diversity indices i.e. Weighted UniFrac, Unweighted UniFrac and Bray-Curtis, were generated in Qiime. The OTU table was taxonomically classified (classify.seqs) to genus level by using Mothur and the RDP v11.4/trainset 14 database (Wang *et al*., 2007). In order to further classify the OTUs to species level Spingo database was used (Allard *et al*., 2015).

Statistical Analysis of microbiota composition: The R package (R Core Team) was used for the generating taxa relative abundance from the rarified reads, for the further analysis and visualisation of the UniFrac distances, for the visualisation of the alpha diversity and for statistical analysis. A Kruskal-Wallis with Dunn's post hoc test and p adjusted value with the Benjamini-Hochberg method was applied in R in order to detect significant differences in the microbiota composition due to

substrate fermentation after 16 h and 24 h at Order, Family, Genus and Species level and for the alpha diversity indices. The Kruskal-Wallis test was applied in order to detect significant differences due to the effect of the substrate per donor, across all donors and MCC100 per time point and per substrate. Values were considered significant when Kruskal-Wallis p value was ≤ 0.05, p_{adj} value ≤ 0.05 and Dunn's post hoc test p_{adj} value (for readability denoted as q_{adj}) were ≤ 0.05. Significant trends in data were considered when Kruskal-Wallis p value was ≤ 0.5, p_{adj} value ≤ 0.1 and Dunn's post hoc test value p_{adj} (for readability denoted as q_{adj}) was ≤ 0.1.

Short Chain Fatty Acid Analysis: High performance liquid chromatography (HPLC) was used in order to detect acetate, propionate and butyrate production, as described before (Chapter 2; Ntemiri *et al.,* 2017). Slurry retrieved from the fermenter vessels was centrifuged for 30 min and the supernatant was filtered through 0.2 μm filter. For the SCFA analysis 1 ml was loaded in an Agilent 1200 HPLC system with a refractive index detector. An Agilent Hi-Plex H 300x7.7mm column was used with 0.01N H_2SO_4 elution fluid, at a flow rate of 0.6 mL/min and column temperature at 65°C. For the visualization and analysis of SCFA levels, Graph Pad Prism 5 Software was used. A Kruskal-Wallis non-parametric test and Dunn's post hoc test were applied in order to detect significant differences in SCFA production per donor per substrate at 24 h. The same test was applied in order to detect significant differences in combined SCFA production in the faecal microbiota of all donors aggregated at 24 h.

4.4 Results

Blueberry responses of Bifidobacteriaceae, Bacteroidaceae and Lactobacillales depend on baseline abundance

We have previously described the compositional and phylogenetic differences in the faecal microbiota of healthy or frail older subjects (COM type microbiota EM425 and EM278, and LS type microbiota EM297 and EM704, respectively) (Jeffery *et al*., 2015; Ntemiri *et al*., 2017; Chapters 2 and 3 this thesis). Significant differences between the two types of microbiota include the enrichment in Lachnospiraceae and Ruminococcaceae in the COM type faecal microbiota composition and the enrichment in Proteobacteria observed in LS type microbiota. The LS type microbiota is also characterised by lower phylogenetic diversity compared to the community type (**S Table 10**). Principal coordinates analysis (PCoA) of the microbiotas at 0 h showed the differential clustering between COM and LS type (**S Figure 8**).

Supplementation with the prebiotic MIX sustained the highest microbiota diversity across all faecal microbiotas (**S Figure 9** and **10)**. Principal coordinates analysis (PCoA) of the microbiotas at the end of the fermentation (24 h) showed that the microbiotas clustered predominantly by donor and microbiota type and to a lesser extent by supplementation (**S Figure 11 A** and **B**).

At baseline, the COM type microbiota of donor EM278 was naturally abundant in Bifidobacteriales/Bifidobacteriaceae and Bacteroidales/Bacteroidaceae; approximately 25% combined relative abundance (**Figures 21** and **22**). Blueberry supplementation (BB) resulted in increased Bifidobacteriaceae relative abundance in the composition of the EM278 COM faecal microbiota whereas prebiotic MIX supplementation increased the Bacteroidaceae abundance after 24 h fermentation. Supplementation with combined blueberry and prebiotic mix (BB.MIX) resulted in the concomitant increase of both Bifidobacteriaceae and Bacteroidaceae relative

abundance; the combined relative abundance increased to approximately 67% (**Figure 22** and **Table 9**). The Bifidobacteriaceae were represented by *Bifidobacterium bifidum*, *Bifidobacterium longum* and other unclassified *Bifidobacterium* taxa whereas the major Bacteroidaceae representative taxa after 24 h MIX or BB.MIX supplementation were *Bacteroides uniformis* and *Bacteroides caccae* (Species level information for COM type microbiota in **S Table 13**). Another faecal microbiota enriched in Bifidobacteriacea and Bacteroidaceae was the LS faecal microbiota from donor EM297 (approximately 20% combined abundance). Bifidobacteriaceae in the EM297 LS type microbiota were not responsive to blueberry supplementation and the baseline abundance (approximately 2%) decreased across all supplementation regimes (**Figure 22**). Bacteroidaceae in the LS microbiota EM297 were predominantly responsive to BB.MIX supplementation whereas across the COM type microbiota EM425 and LS type microbiota EM704 the Bacteroidaceae were predominantly responsive to the prebiotic MIX supplementation (**Figure 22).**

Lactobacillales were highly responsive to blueberry supplementation (BB or BB.MIX) predominantly in the EM425 COM type faecal microbiota and in the EM704 LS type microbiota whereas inclusion of the prebiotic MIX in the blueberry supplemented medium (BB.MIX) promoted the growth of a wider array of taxa resulting in a lower scale relative abundance of Lactobacillales after 24 h fermentation (**Figure 21**). In the EM425 COM microbiota, BB supplementation increased the Lactobacillales relative abundance from approximately 0.5% (0 h) to 33% (p_{adj} & q_{adj} <0.1) at 24 h (comparison data at Order level for COM and LS microbiota in **S Table 11** and **12,** respectively). In the EM704 LS microbiota the Lactobacillales relative abundance increased after BB supplementation from 0.1 % at

0 h to 72 % at 24 h (p_{adj} & q_{adj} <0.1) (**Figure 21**). In both EM425 and EM704 the taxon *Streptococcus* (unclassified) was the dominant representative of the Lactobacillales (**S Tables 13** and **14**).

In the EM297 LS microbiota the Lactobacillales was represented by the taxon *Enterococcus* (unclassified) (**S Table 14)**. The taxon relative abundance was significantly (trend) increased by both BB supplementation (0 h: 2.5%, 24 h: 9% p_{adj} & q_{adj}<0.1) and BB.MIX supplementation (0 h: 1.5%, 24 h: 9.6%, p_{adj} & q_{adj} ≤0.1). The responsiveness of enterococci to blueberry supplementation was of a lower scale compared to the streptococci responsiveness, and prebiotic MIX supplementation promoted a higher increase in the relative abundance of enterococci in the EM297 faecal microbiota (**Figures 22**).

Blueberry supplementation increased the abundance of Enterobacteriaceae across all donors faecal microbiota

Blueberry supplementation (BB) increased the relative abundance of the Proteobacteria/Enterobacteriaceae represented by *Escherichia/Shigella* taxa across all donors faecal microbiotas whereas supplementation of fermentation medium with prebiotic MIX (MIX or BB.MIX) balanced the overrepresentation of the Enterobacteriaceae family observed with BB supplementation (**Figure 22**). The increase in the enterobacteria was partly independent the baseline Proteobacteria abundance. For example, in the COM type EM425 microbiota the low in abundance Enterobacteriaceae increased from <0.05% to 40% at 24 h (**Table 9**) whereas in the EM297 LS type faecal microbiota that was significantly enriched in Proteobacteria, BB supplementation resulted in the highest Enterobacteriaceae relative abundance increase from approximately 9% to 69% at 24 h (p_{adj} & q_{adj} <0.1) (**Table 10**). A

comparatively lower scale increase in the enterobacteria relative abundance was observed in the EM704 LS type microbiota; combined BB and MIX supplementation resulted in the highest abundance increase of the enterobacteria (**Table 10**).

The prebiotic MIX retained relatively higher Lachnospiraceae and Ruminococcaceae combined abundance whereas *Faecalibacterium prausnitzii* showed some responsiveness to BB supplementation

The high Lachnospiraceae and Ruminococcaceae relative abundance observed in the COM type microbiotas (approximately 66% combined abundance in both EM278 and EM425) was not retained across any of the three supplementation regimes (**Figure 22).** Supplementation with prebiotic MIX alone retained the highest portion of the Lachnospiraceae abundance in COM type microbiotas EM278 and EM425 and LS type EM704, whereas supplementation with BB and MIX combined retained the highest Lachnospiraceae abundance in the LS type microbiota EM297 (**Tables 9** and **10**). The taxon *Anaerostipes hadrus* was highly responsive to co-supplementation in both COM microbiotas (**S Table 13**).

Similarly, Ruminococcaceae abundance was partially retained with MIX and BB.MIX supplementation in COM type EM425 and LS type EM704 microbiotas (**Tables 9** and **10**), whereas the Ruminococcaceae in the COM microbiota EM278 were highly responsive to BB supplementation; the same responsiveness was not observed after MIX or BB.MIX supplementation (**Figure 22**, **TABLE 9**).

Faecalibacterium prausnitzii was the predominant taxon of the Ruminococcaceae family (approximately 13% relative abundance) in the EM278 microbiota after BB supplementation (**S Table 13**). In EM425 microbiota, the taxon *F. prausnitzii* was the predominant member of the Ruminococcaceae family after BB or MIX

supplementation (2.5% and 5% relative abundance respectively), whereas the taxon *Gemmiger formicilis* of the Ruminococcaceae was dominant after BB.MIX or MIX supplementation (approximately 7% and 2% relative abundance respectively) (**S Table 13**). In EM704 LS faecal microbiota, of unclassified taxa were the dominant representatives of the Ruminococcaceae family (**S Table 14**).

Blueberry supplemented prebiotic medium promoted the abundance of Peptostreptococcaceae and Clostridiaceae 1 when abundant at baseline and irrespective of microbiota type.

In the EM425 COM type faecal microbiota where the Peptostreptococcaceae family was present with approximately 3% relative abundance at baseline, BB and BB.MIX but not MIX supplementation alone resulted in increased relative abundance during the first 16 h of BB (4.5% relative abundance) and BB.MIX (3% relative abundance); however, the abundance of the taxon was not retained until the fermentation end (**Figure 22, Table 9**). In the LS type faecal microbiota EM704 where the Peptostreptococcaceae was present with approximately 11% relative abundance at baseline, BB or MIX supplementation alone could not retain Peptostreptococcaceae relative abundance whereas BB.MIX supplementation resulted in increased relative abundance at 24 h (**Figure 22, Table 10**). *Clostridium glycolicum* and other unclassified taxa were the dominant *Clostridium* cluster XI representatives of the Peptostreptococcaceae at 24 h (**S Table 14**). Abundant baseline Clostridiaceae 1 (represented by *Clostridium* sensu stricto taxa) increased in abundance with BB.MIX and MIX supplementation in both the COM type microbiota EM425 and the LS type EM704 microbiota whereas when non-abundant

at baseline as in LS type EM297 microbiota, the abundance increased after MIX supplementation (**Figure 22, Tables 9** and **10**).

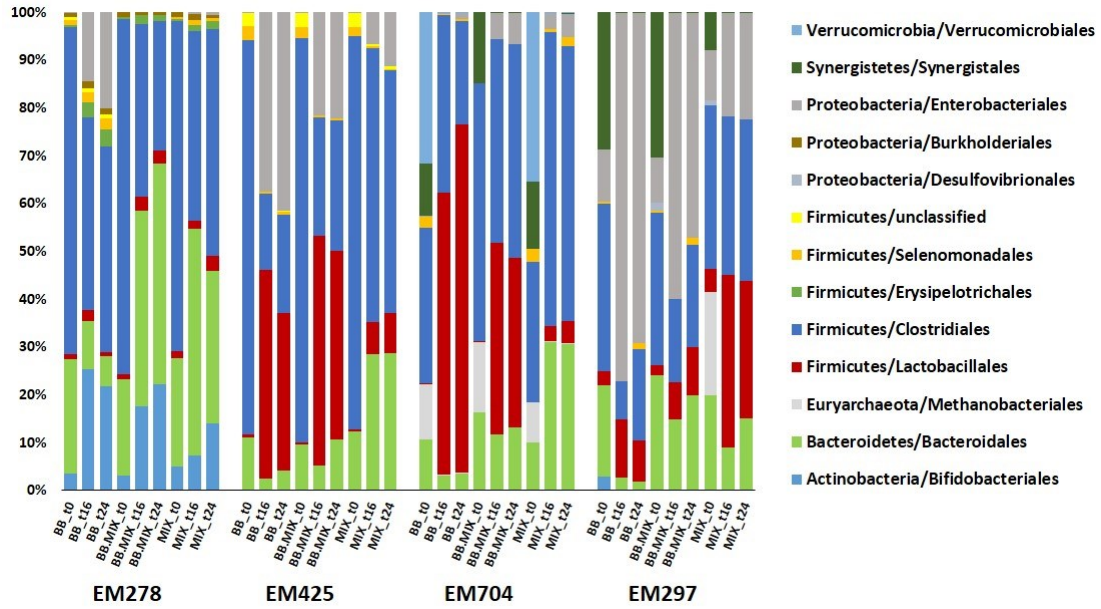


Figure 21 Order level development of the faecal microbiota from COM donors (EM278, EM425) and LS donors (EM704, EM297). Orders present with relative abundance ≥1% are shown. Substrate conditions are basal medium supplemented with: BB: blueberry powder; BB.MIX: blueberry powder and prebiotic mix of carbohydrates; MIX: mix of prebiotic carbohydrates. Time points: 0 h, 16 h and 24 h of fermentation.

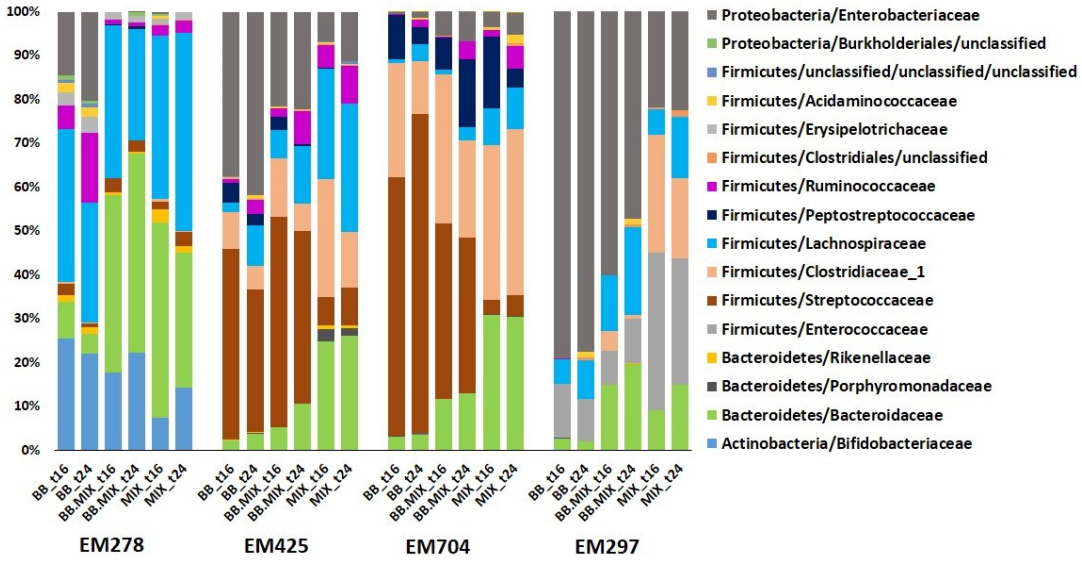


Figure 22 Family level development of the faecal microbiota from COM donors (EM278, EM425) and LS donors (EM704, EM297). Families present at ≥1% are shown. Substrate conditions are basal medium supplemented with: BB: blueberry powder; BB.MIX: blueberry and prebiotic mix of carbohydrates; MIX: mix of prebiotic carbohydrates. Time points: 16 h and 24 h of fermentation.

Table 9 Development of the composition and comparison between time points of the relative abundance and comparison between time points of the relative abundance of dominant families after 24 h fermentation with COM faecal microbiota (EM278, EM425). BB: blueberry powder supplementation; BB.MIX: blueberry powder and prebiotic carbohydrate mix supplementation; MIX: prebiotic carbohydrate mix supplementation. Missing values indicate that the relevant taxon had <1% abundance in all vessels (two or three) summed for mean abundance. The mean value of the relative abundance per time point is shown.

	EM278						EM425					
	BB		BB.MIX		MIX		BB		BB.MIX		MIX	
	t0	t24	t0	t24	t0	t24	t0	t24	t0	t24	t0	t24
Bifidobacteriaceae	3.5	21.6	3.0	21.9	4.9	13.9[a]	-	-	-	-	-	-
Bacteroidaceae	20.0	4.5	16.9	44.8	19.1	29.9[a]	5.8	3.7	4.6	10.6	6.8	26.1
Porphyromonadaceae	-	-	-	-	-	-	2.7	0.3	-	-	2.7	1.7
Rikenellaceae	1.7	1.5	1.3	0.4	1.8	1.5	1.6	0.1	-	-	1.6	0.6[a]
Streptococcaceae	1.1	0.9	1.0	2.6	1.3	3.1	0.5	32.1[a]	0.4	39.1	0.4	8.5
Clostridiaceae_1	0.1	0.2	-	-	0.1	0.1	3.1	5.3	3.9	6.2	2.7	12.6[a]
Lachnospiraceae	43.7	26.7	49.5	25.0	43.4	43.8	44.4	9.3[a]	42.8	13.0	46.4	29.0[a]
Peptostreptococcaceae	-	-	0.3	0.6	-	-	3.2	2.5	3.5	0.4	2.9	0.2
Ruminococcaceae	20.1	15.7	19.2	0.8	21.0	2.9	21.2	3.2[a]	22.8	7.6	19.4	8.5[a]
Clostridiales unclas.	2.7	0.1	2.5	0.1	2.5	0.2[a]	3.8	0.2[a]	4.0	0.1	3.6	0.2
Erysipelotrichaceae	0.3	3.5	0.4	1.4	0.5	1.7[a]	-	-	-	-	-	-
Acidaminococcaceae	0.4	2	-	-	0.2	0.6[a]	2.6	0.7[a]	2.0	0.4	1.8	0.3[a]
Firmicutes/unclassified	0.8	1	-	-	-	-	2.6	0.2[a]	2.7	0.02	2.7	0.6[a]
Enterobacteriaceae	0.01	20	-	-	0.01	0.5	0.04	41.3	0.03	22.0	0.03	11.1[a]

* Statistically significant result, Kruskal-Wallis test result p≤0.05, padj≤0.05 with Dunn's test qadj j≤0.05
a Statistical trend, Kruskal-Wallis test result p≤0.05, padj≤0.1, with Dunn's test qadj ≤0.1.

Table 10 Development of the composition and comparison between time points of the relative abundance of dominant families after 24 h fermentation with LS faecal microbiota (EM704, EM297). BB: blueberry powder supplementation; BB.MIX: blueberry powder and prebiotic carbohydrate mix supplementation; MIX: prebiotic carbohydrate mix supplementation. Missing values indicate that the relevant taxon had <1% abundance in all vessels (two or three) summed for mean abundance. The mean value of the relative abundance per time point is shown.

	EM704						EM297					
OTUU	**BB**		**BB.MIX**		**MIX**		**BB**		**BB.MIX**		**MIX**	
Classification	**t0**	**t24**	**t0**	**t24**	**t0**	**t24**	**t0**	**t24**	**t0**	**t24**	**t0**	**t24**
Bifidobacteriaceae	-	-	-	-	-	-	2.3	0.02	-	-	-	-
Bacteroidaceae	6.0	3.5	6.4	12.9	5.4	29.9	12.9	1.8	13.9	18.5	15.1	14.6
Porphyromonadaceae	3.2	0.1	-	-	3.5	0.3	1.4	0.04	1.5	0.1[a]	2.7	0.02
Rikenellaceae	-	-	-	-	1.0	0.04	1.2	0.02	1.3	0.02	1.5	0.01
Enterococcaceae	-	-	-	-	-	-	1.1	8.6[a]	0.5	9.6[a]	1.6	28.1
Streptococcaceae	0.1	72.0[a]	0.1	35.0	0.1	4.5[a]	-	-	-	-	-	-
Clostridiaceae_1	10.1	11.8	11.1	21.7	9.2	37.3	-	-	0.01	0.9[a]	0.01	17.9
Lachnospiraceae	3.3	3.7	3.2	3.1	3.2	9.3	15.3	7.7	11.9	18.7	17.0	13.6
Peptostreptococcaceae	11.7	4.0	13.4	15.1	10.3	4.3[a]	-	-	-	-	-	-
Ruminococcaceae	3.6	1.6[a]	3.2	4.0	3.7	5.2a[a]	11.2	0.1	8.7	0.1	13.7	0.1
Clostridiales unclas.	1.8	0.1	2.1	0.1	1.9	0.6[a]	2.0	0.5[a]	1.5	0.5[a]	2.3	1.4
Acidaminococcaceae	2.3	0.3[a]	-	-	2.6	1.9[a]	0.3	1.1	0.2	1.3	-	-
Enterobacteriaceae	0.1	1.2	0.04	6.6	0.1	4.9	8.8	68.5[a]	6.6	44.3[a]	10.2	21.9

[*] Statistically significant result, Kruskal-Wallis test result $p \leq 0.05$, $p_{adj} \leq 0.05$ with Dunn's test $q_{adj} \leq 0.05$
[a] Statistical trend, Kruskal-Wallis test result $p \leq 0.05$, $p_{adj} \leq 0.1$, with Dunn's test $q_{adj} \leq 0.1$

Blueberry supplemented prebiotic medium sustained high microbiota diversity and *Bacteroides* taxa abundance in the artificial consortium MCC100 compared to the other supplementation regimes

The artificial consortium MCC100 composition was designed in order to mimic the faecal microbiota composition of a healthy adult. The analysis of the relatedness by Weighted Principal Coordinates Analysis (PCoA) of the microbiota composition in the bacterial consortium and faecal microbiotas showed that at 0 h the MCC100 grouped with the community type faecal microbiotas separating from the LS type microbiotas (PCoA information in **S Figure 8 A** and **B**), as expected from its design. The distance between PCoA positions of some MCC100 microbiota (**S Figure 8 A**) was associated with the presence of OTUs assigned to chloroplasts deriving from the blueberry supplementation. Therefore, it was not considered a true compositional difference between the MCC100 preparations inoculated at 0 h.

Supplementation of fermentation medium with BB.MIX sustained similar or higher levels of alpha diversity to the prebiotic MIX supplementation in the MCC100 microbiota (**Table 11**). The Shannon and Simpson indices were similar after BB.MIX or MIX supplementation, whereas species richness indices (Chao1, Observed species and PD whole tree) were higher after BB.MIX supplementation. BB supplementation alone sustained the lowest diversity among the supplementation regimes.

Supplementation of the MCC100 fermentation with BB.MIX was more effective in sustaining the abundance of Bacteroidetes compared to prebiotic MIX supplementation alone (**Figure 23 A**). Bacteroidetes relative abundance increased from approximately 12% to 52% over the 24 h of BB.MIX fermentation (p_{adj}=0.05,

q_{adj}=0.02) whereas MIX supplementation resulted in a decrease in the relative abundance of Bacteroidetes during the first 16 h; the relative abundance of the phylum subsequently increased to an average of 45% (24 h, p_{adj}=0.04, q_{adj}=0.1). The Bacteroidaceae family were the main Bacteroidetes representatives (**Figure 23 B**). The average relative abundance of the Bacteroidaceae increased from approximately 6% (16 h) to 46% (24 h) and from 6% (0 h) to 52% (24 h) after MIX and BB.MIX supplementation respectively (**Figure 23 B**). Supplementation with BB had a relatively lower scale impact on the Bacteroidaceae (**Figure 23 B**). *Bacteroides fragilis* and *Bacteroides thetaiotaomicron* were the two most responsive taxa of the Bacteroidaceae family across supplementation regimes (**Table 12**). After 24 h BB.MIX supplementation *B*. *fragilis* relative abundance increased from 0.5% to 24%, (p_{adj}=0.04, q_{adj}=0.01) whereas after MIX supplementation the abundance was approximately 38%, ($p_{adj=}$0.05 q_{adj}=0.01). The highest relative abundance increase for *B*. *thetaiotaomicron* was observed after BB.MIX supplementation from approximately 1% to 26% (p_{adj}=0.04, q_{adj}=0.01). The taxon *Bacteroides caccae* was responsive to BB.MIX supplementation and its average relative abundance had a significant increasing trend from 0.4% to 1% after 24 h fermentation, (p_{adj}=0.04, q_{adj}=0.1).

Firmicutes tended to decrease across the three supplementation regimes whereas MIX supplementation sustained a relatively higher portion of the Firmicutes abundance (**Figure 23 A**). The differences in the relative abundance of Firmicutes taxa observed at baseline (**Table 12**) between the three supplementation conditions can be attributed to some variation introduced during the preparation of the consortium; gDNA extraction and sequencing bias may have amplified this variation

resulting in increased relative representation of the phylum at baseline of the MIX supplementation regime.

Lachnospiraceae relative abundance showed a decreasing trend across all supplementation regimes during the first 16 h of fermentations; the abundance of the Lachnospiraceae tended to increase thereafter (**Figure 23 B**). Twenty-four h BB.MIX supplementation retained a comparatively high Lachnospiraceae relative abundance (14%) compared to MIX (11%) and BB supplementation (6%). The relative abundance of Lachnospiraceae taxon *Clostridium symbosium* reached its highest abundance after BB supplementation followed by BB.MIX supplementation whereas *Coprococcus comes* retained the highest abundance after BB.MIX supplementation (approximately 8 %) (**Table 12**). Other dominant Firmicutes taxa responsive to blueberry supplementation belonged to the *Clostridium* cluster XI of the Peptostreptococcaceae family (**Table 12**). The Lactobacillales were more responsive to the MIX supplementation. The taxon *Enterococcus* was the main representative of the Lactobacillales and increased in relative abundance from <0.1% (0 h) to approximately 21% (p_{adj}=0.05, q_{adj}=0.01) during the first 16 h of MIX supplementation; a similar but to a lesser scale response was observed with BB and BB.MIX supplementation (**Table 12**).

The Proteobacteria relative abundance increased over the first 16 h across all supplementation regimes and tended to decrease thereafter; the highest relative abundance was observed after BB supplementation (60% average relative abundance at 24 h; p_{adj}=0.09, q_{adj}=0.08) (**Figure 23 A**). Supplementation with prebiotic MIX or BB.MIX had a similar effect on the abundance of the main Proteobacteria taxa that

belonged to the *Escherichia*/*Shigella* group, i.e., approximately 20% relative abundance after 24 h fermentation (**Table 12**).

Table 11 MCC100 alpha diversity indices after 24 h fermentation with the selected substrates. BB: blueberry powder supplementation; BB.MIX: blueberry powder and prebiotic carbohydrate mix supplementation; MIX: prebiotic carbohydrate mix supplementation.

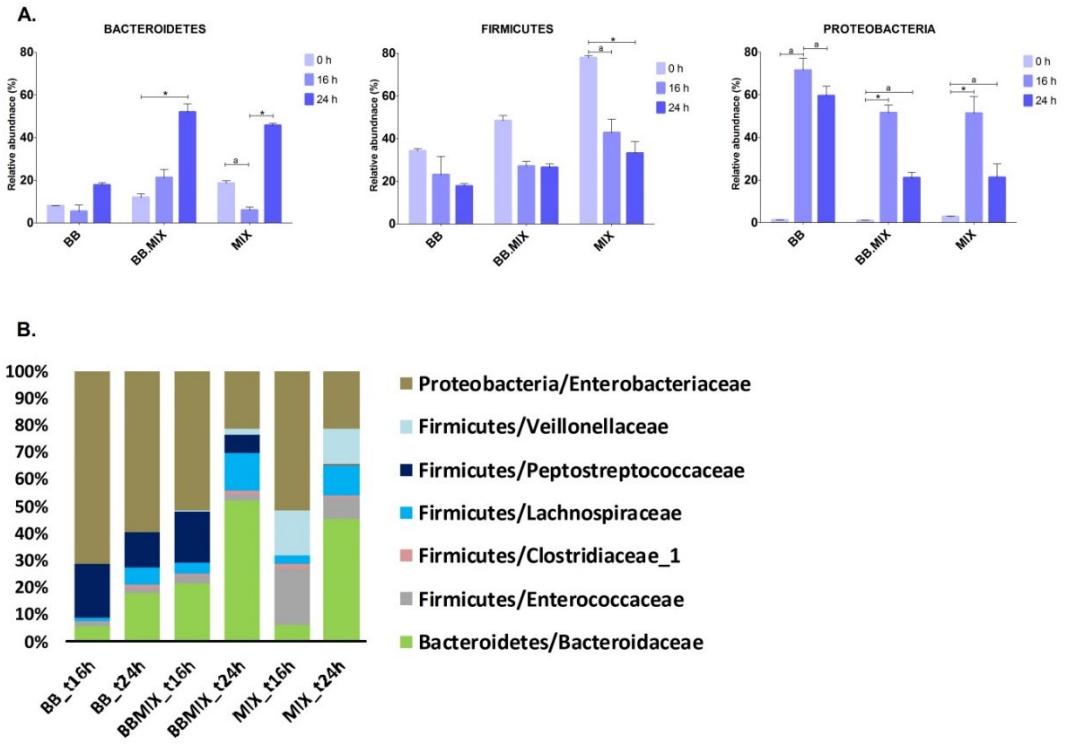
	Mean alpha diversity value at 24 h with indicated substrate		
Alpha Diversity Indices	**BB**	**BB.MIX**	**MIX**
Shannon	2.0	2.9	2.7
Simpson	0.6	0.8	0.8
PD whole tree	3.3	4.4	3.7
Chao1	34.8	68.2	45.4
Observed species	32.0	43.0	35.0

Table 12 Development of the relative abundance at species level in the MCC100 after 24 h fermentation and comparison by time point. BB: blueberry powder supplementation; BB.MIX: blueberry powder and prebiotic carbohydrate mix supplementation; MIX: prebiotic carbohydrate mix supplementation. Missing values indicate that the relevant taxon had <1% abundance in all vessels (two or three) summed for mean abundance.

	MIX			BB. MIX			BB		
OTU Classification	**t0**	**t16**	**t24**	**t0**	**t16**	**t24**	**t0**	**t16**	**t24**
Bacteroides/Bacteroides caccae	-	-	-	0.4	0.1	1.0*	-	-	-
Bacteroides/Bacteroides dorei	4.1	0.01*	0.1	-	-	-	-	-	-
Bacteroides/Bacteroides fragilis	1.0	5.9	37.8*	0.5	14.7	24.3*	0.5	0.9	2.1
Bacteroides/Bacteroides thetaiotaomicron	1.81	0.1	7.5*	1.0	6.4	26.3*	1.0	4.5	15.4
Odoribacter/Odoribacter splanchnicus	-	-	-	2.9	0.004[a]	0.01	-	-	-
Enterococcus/unclassified	0.1	20.8*	7.6	0.01	2.8	2.2	0.02	1.7	1.9
Clostridium sensu stricto/ Clostridium baratii	0.03	1.9	1.0	0.04	1.2	1.3	0.01	0.1	1.3[a]
Clostridium XlVa/ Clostridium hathewayi	0.3	0.2	2.1	-	-	-	-	-	-
Anaerostipes/Anaerostipes hadrus	-	-	-	-	-	-	6.5	0.02	0.2
Clostridium XlVa/Clostridium symbiosum	0.1	1.0	2.4*	0.04	0.8	3.4*	0.04	1.5	5.0[a]
Clostridium XlVa/unclassified	0.1	0.03	0.6*	-	-	-	-	-	-
Coprococcus/Coprococcus comes	25.5	0.5*	1.2[a]	13.5	3.0*	7.8[a]	9.6	0.1	0.1
Roseburia/Eubacterium rectale	-	-	-	2.7	0.02	0.1	1.9	0.0	0.01
Lachnospiraceae/ unclassified	5.3	1.6	4.9	3.1	0.1*	0.7	2.6	0.02*	0.1
Clostridium XI/unclassified	-	-	-	0.01	18.2*	6.2	0.0	16.8	11.2
Veillonella/Veillonella dispar	0.03	16.0	12.7	0.01	0.1	2.2	-	-	-
Escherichia/Shigella/ unclassified	2.6	51.3*	21.2	0.8	51.5*	21.0	1.1	71.4[a]	59.5

*Statistically significant result, Kruskal-Wallis test result $p \leq 0.05$, $p_{adj} \leq 0.05$ with Dunn's test $q_{adj} \leq 0.05$.

[a] Statistical trend, Kruskal-Wallis test result $p \leq 0.05$, $p_{adj} \leq 0.1$, with Dunn's test $q_{adj} \leq 0.1$.



5229

5230 **Figure 23 Development of the MCC100 composition after 24 h fermentation.**
5231 The relative abundance of the dominant phyla Bacteroidetes, Firmicutes and
5232 Proteobacteria (**A**) and the relevant families (**B**) is shown for time points 0 h, 16 h
5233 and 24 h. BB: blueberry powder supplementation; BB.MIX: blueberry powder and
5234 prebiotic carbohydrate mix supplementation; MIX: prebiotic carbohydrate mix
5235 supplementation. Only families present with ≥1% relative abundance are shown. "*"
5236 statistically significant: Kruskal-Wallis test result $p \leq 0.05$, $p_{adj} \leq 0.05$, Dunn's test
5237 $q_{adj} \leq 0.05$. "a" statistical trend: Kruskal-Wallis test result $p \leq 0.05$, $p_{adj} \leq 0.1$, Dunn's
5238 test $q_{adj} \leq 0.1$.

5239 **Blueberry supplemented prebiotic medium resulted in increased short chain**
5240 **fatty acid production across the four faecal microbiotas and MCC100.**

5241 The production levels of the SCFAs acetate, propionate and butyrate levels in the
5242 community type faecal microbiotas (EM278, EM425) and the longstay type

(EM297, EM704) after 24 h fermentation are shown in **Figure 25 A** and **B** respectively. Combined supplementation of fermentation medium with blueberry powder and prebiotic carbohydrates (BB.MIX) resulted in increased SCFA production across all donors faecal microbiota irrespective of community or longstay type and in comparison to prebiotic (MIX) or blueberry (BB) supplementation alone. The effect of substrate supplementation on combined SCFA levels in the aggregated across the four donors faecal microbiota is shown in **Figure 25 C**. BB.MIX supplementation resulted in significantly higher SCFA levels (mean 210 μmol mean SCFA per g of faecal pellet) compared to BB (mean 124 μmol per g of faecal pellet, $p<0.005$) and MIX (mean 150 μmol per g of faecal pellet, $p<0.0005$). Similarly, in the MCC100 microbiota BB.MIX supplementation resulted in higher SCFA levels after 24 h fermentation compared to the other supplementation regimes (**Figure 25 D**). Acetate and butyrate production were significantly higher after BB.MIX supplementation (mean 66 μmol per g of bacterial pellet, $p<0.05$ and 14 μmol per g of bacterial pellet, $p<0.05$ respectively) compared to MIX supplementation (5.5 and 7 μmol per g of bacterial pellet respectively).

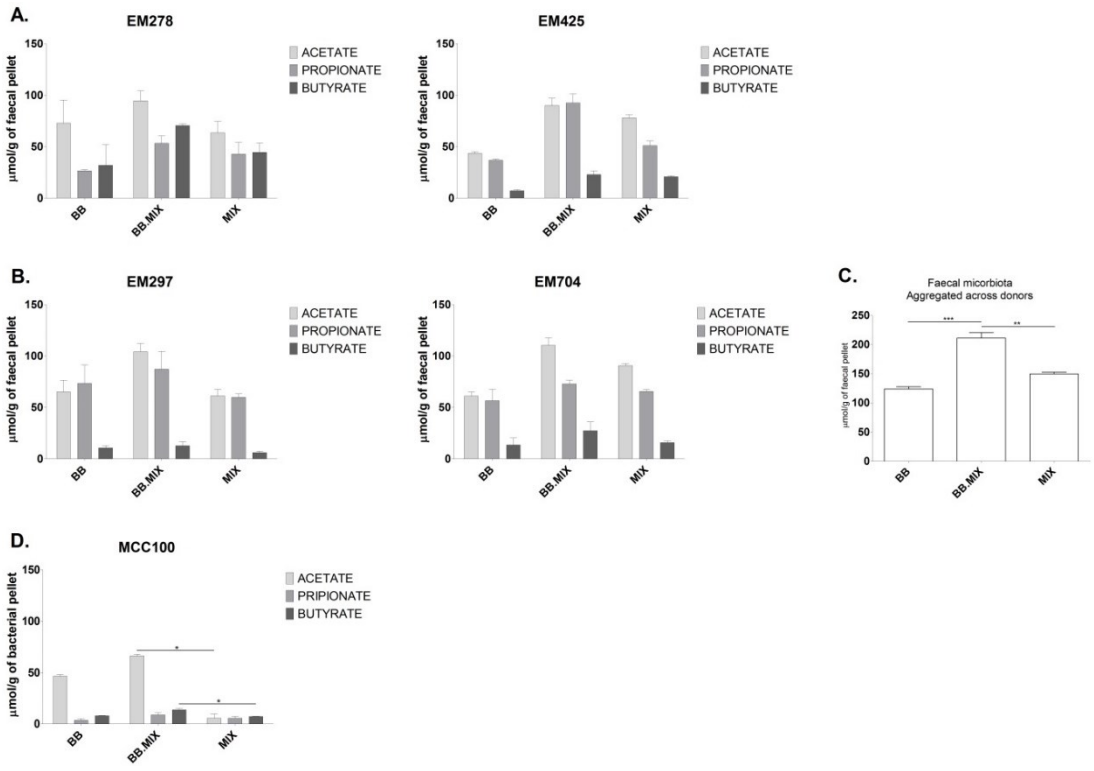


Figure 24 Short chain fatty acid (SCFA) production by the faecal microbiota and artificial consortium MCC100 after 24 h fermentation with selected substrates. The concentration (µmol/g of faecal pellet) of acetate, propionate and butyrate in the fermentation fluid from the COM type (EM278 and EM425) and LS type (EM297, EM704) faecal microbiotas and consortium MCC100 is shown in **A**, **B** and **D** respectively. The combined SCFA production in the aggregated (i.e. across all donors faecal microbiotas) is shown in panel **C**. Kruskal-Wallis test with Dunn's post hoc test was applied to compare the SCFA levels at 24 h. *** $p \leq 0.0005$, ** $p \leq 0.005$, * $p \leq 0.05$. BB: blueberry powder supplementation; BB.MIX: blueberry powder and prebiotic supplementation; MIX: prebiotic carbohydrate mix supplementation.

4.5 Discussion

Baseline-dependent prebiotic effect of whole blueberry extract on the gut microbiota of older donors

In this study we explored the modulatory effect of the rich in polyphenols and dietary fibre whole blueberry powder *in vitro* on the faecal microbiota of older subjects. Blueberry powder was used either as the sole fermentation substrate or in combination with prebiotics. By testing the supplementation regimes and analysing its effect on individual faecal microbiotas we observed the differential prebiotic potential of blueberries that was broadly determined by the baseline microbiota composition. Differential responsiveness to prebiotic treatment due to inter-individual compositional differences in the gut microbiota has been repeatedly reported calling for personalized strategies towards restoring the dysbiotic gut microbiota and retaining health (Duncan and Flint, 2013). Notwithstanding, due to the increasing understanding of the ecological forces that assemble the gut microbiota communities and the role of certain health-related taxa in disease, prebiotics remain a promising generic therapeutic approach (Scott *et al*., 2015; Bashan *et al*., 2016).

The presence of a dominant *Bifidobacterium* and *Bacteroides* population responsive to blueberry supplementation differentiated the EM278 community type microbiota from the other community type and the longstay type microbiotas. In the case of the community type EM278 bifidobacteria and *F. prausnitzii* relative abundance increased when blueberries were the sole fermentation substrate whereas bifidobacteria and *Bacteroides* increased after co-supplementation with blueberries and prebiotics suggesting extensive cross-feeding between bifidobacteria and either

butyrate-producers like *F. prausnitzii* or *Bacteroides* taxa. Cross-feeding between butyrate-producing bacteria like *F. prausnitzii* and acetate-producing bifidobacteria have been extensively described before (Moens *et al*., 2016). Strain-dependent cross-feeding phenomena between *Bifidobacterium* and *Bacteroides* taxa have also been described (Rios-Covian *et al*., 2013). *In vitro* responsiveness of bifidobacteria predominantly and to a lesser extent of *F. prausnitzii* to polyphenols-rich extracts from fruits like blueberries, pomegranate and apple, has been reported before (Molan *et al*., 2009; Balionska *et al*., 2010; Guergoletto *et al*., 2016; Koutsos *et al*., 2017). There are well-documented health-claims for *bifidobacteria, F. prausnitzii* and *Bacteroides* taxa. Decreased relative abundance of *F. prausnitzii* population associates to IBD whereas bifidobacteria abundance decreases with ageing (Miquel *et al*., 2013; Arboleya *et al*., 2016). Various *Bacteroides* are next generation probiotic candidates (El Hage *et al*., 2017). Interestingly, although the combined supplementation favoured other dominant bacterial groups and inhibited *F. prausnitzii* from accessing the blueberries substrate, the resulting SCFA production was the highest among supplementation regimes, including the highest production of butyrate indicating that although *F. prausnitzii* population was not sustained other butyrate producing Lachnospiraceae like *A. hadrus* may have benefitted from cross-feeding phenomena with bifidobacteria as reported before (Belenguer *et al*., 2006).

Increased SCFA production was observed across all faecal microbiotas and MC100 after co-supplementation compared to prebiotics supplementation alone. Although acetate and propionate were the major SCFAs, butyrate production profile was promoted by the co-supplementation. In animal trials diet combining strawberry polyphenols-rich extracts with fructooligosaccharides (FOS) resulted in increased caecal SCFA production especially butyrate suggesting a synergistic beneficial effect

of the two substrates on the GIT health (Singh *et al*., 2017). Others have reported beneficial metabolic effects of similar co-supplementation in murine models but not increased SCFA production (Fotschki *et al*., 2016). The SCFA propionate and butyrate are strongly associated to host health produced mostly by Bacteroidetes and certain Clostridiales respectively, whereas acetate is an abundant SCFA by-product of the colonic bacteria fermentation activity (Louis and Flint, 2017).

Co-supplementation supported a greater production of SCFA which was accompanied by a concomitant increase in alpha diversity indices only in MCC100. Due to its wide repertoire of carbohydrate utilization (Flint *et al*., 2012) *Bacteroides* were among the taxa that increased in abundance after co-supplementation across the faecal microbiotas and especially in MCC100. The *Clostridium* cluster XIVa taxa *Clostridium symbosium* and *Coprococcus comes* were identified as highly responsive to co-supplementation in MCC100. Members of the *Clostridium* cluster XIVa are major butyrate producers and potential candidates for probiotic consortium design (El Hage *et al*., 2017). The synthetic community MCC100 has allowed monitoring the responsive behaviour of selected dominant faecal bacteria to supplementation potentially through what Clavel *et al*. (2017) has described as "functional enrichment via community reduction".

Importantly, Lactobacillales represented here by *Streptococcus* taxa were highly responsive to blueberry supplementation whereas *Enterococcus* taxa demonstrated lower responsiveness. Others have reported *in vitro* responsiveness of Lactobacillales (measured with *Lactobacillus*/*Enterococcus* FISH and qPCR probes) to supplementation with various polyphenols rich extracts (Balionska et al., 2010; Cueva *et al*., 2013; Zhang *et al*., 2016; Gil-Sanchez *et al*., 2017). Importantly, Lee *et*

al. (2018) reported that in mice fed high-fat diet blueberry powder administration resulted in amelioration of the GIT physiology and inflammation that correlated to an increase in Lactobacillales and Proteobacteria abundance. The Lactobacillales is a prominent member of the human gut microbiota; the order harbours probiotic strains but also potential pathobionts (Rajilic-Stojanovic and de Vos, 2014). Enterococci and streptococci have been identified to be involved in polyphenols metabolism (Rowland *et al*., 2018). Streptococci may have readily responded to both the blueberry fruit sugars and the polyphenol content.

Effect of whole blueberry extract on the abundance of Enterobacteriaceae

We observed a relatively high increase in the Gammaproteobacteria with the blueberry supplementation. It has been reported before that Enterobacteriaceae may play a role in polyphenols metabolism (Couteau *et al*., 2001) and increase in Enterobacteriaceae population with a concomitant increase in *Bacteroides* was observed due to tannin-rich diets in murine models (Smith and Mackie, 2004). Recently, Lee *et al*. (2018) reported that in the faecal microbiota of rats fed high-fat diet supplemented with whole blueberry extract the abundance of Gammaproteobacteria, mainly Pasteurellaceae, Fusobacteriaceae and Porphyromonadaceae was increased compared to feeding with high-fat diet or low-fat diet. Proliferation of Proteobacteria and Fusobacteria in the gut microbiota is associated with dysbiosis (Gevers *et al*., 2014; Eklof *et al*., 2017; Litvak *et al*., 2017). In the Lee *et al*. (2018) study the seemingly dysbiotic modulation of the murine faecal microbiota after blueberry supplementation was accompanied with improved GIT function and lowered inflammation markers. Increase in Proteobacteria abundance was observed in the faecal microbiota of rats after Roux-

en Y gastric bypasses and was correlated to postoperative weight loss (Shao *et al*., 2017).

4.6 Conclusion

Importantly, dietary polyphenolic compounds may be bound to dietary fibre impacting the bioavailability and the metabolism of the substrates by the colonic microbiota (Edwards *et al*., 2017). Dietary fibre in polyphenols rich substrates may better support the growth of various taxa such as *Bacteroides* taxa as observed in *in vitro* and in human trials (Eid *et al*., 2014; Henning *et al*., 2017) and is more relevant to human diet. Here, we reported that rich in polyphenols and dietary fibre whole blueberry supplementation can affect certain health-relevant taxa and drive an increased SCFA production in the faecal microbiota of elderly. Blueberries potentially promote elderly health by contributing fibre to the fibrolytic colonic bacterial community thus, increasing the SCFA production that is known to decline with ageing (Duncan and Flint, 2013) and also by contributing phenolic metabolites. The lack of compartmentalisation and the reduction in diversity of the faecal microbiota under *in vitro* conditions significantly impact on microbiota responsiveness to supplementation tests (Guerra *et al*., 2012). The awaited phenolic compound analysis will reveal bioconversion of these compounds by the microbiota during the 24 h fermentation. Follow-up *in vitro* trials with isolated phenolic compounds will elucidate the role of blueberry extracts on gut microbiota modulation. *In vivo* trials will leverage the *in vitro* bias and further elucidate the microbiota modulatory potential of whole blueberries allowing for safe dietary recommendations for the elderly.

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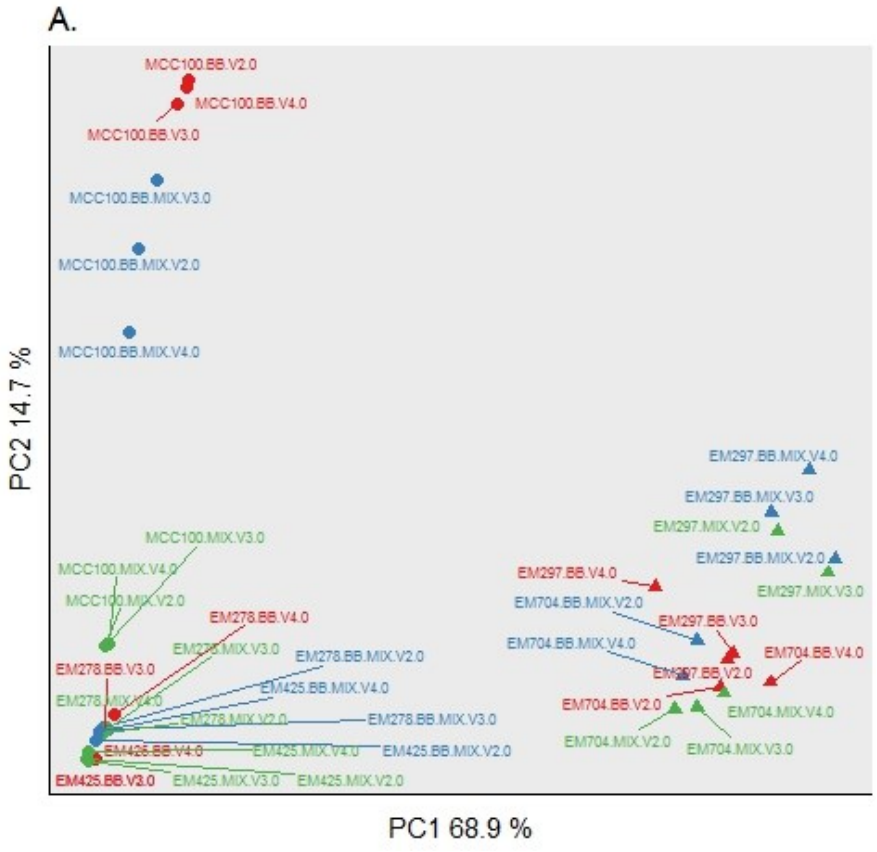
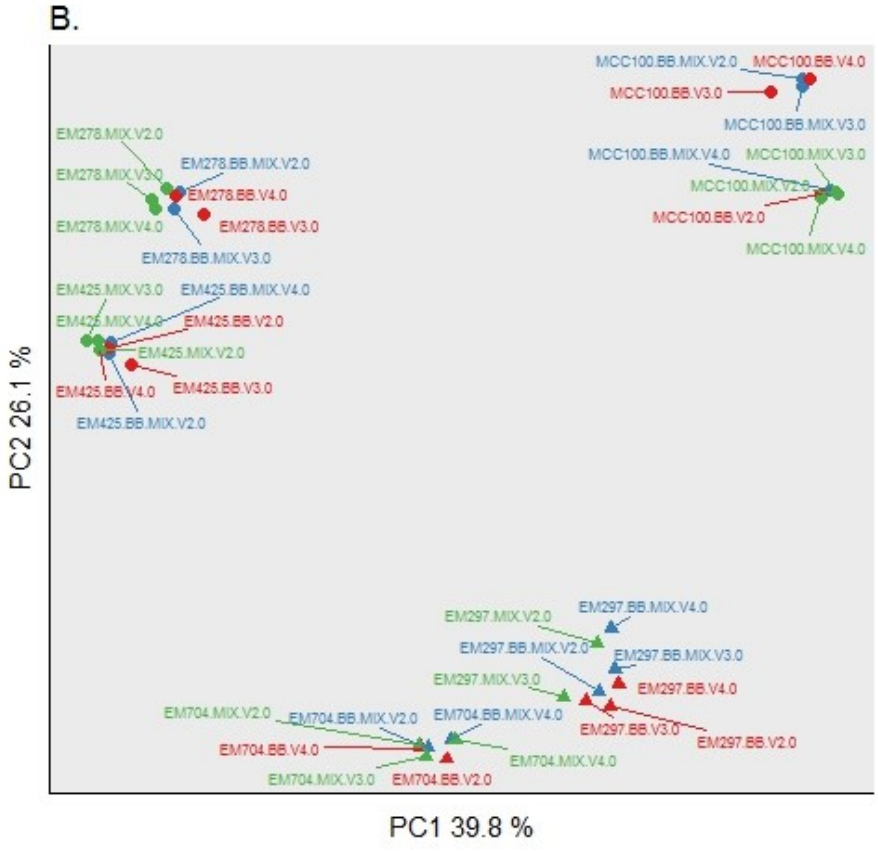
4.10 Supplementary Chapter 4

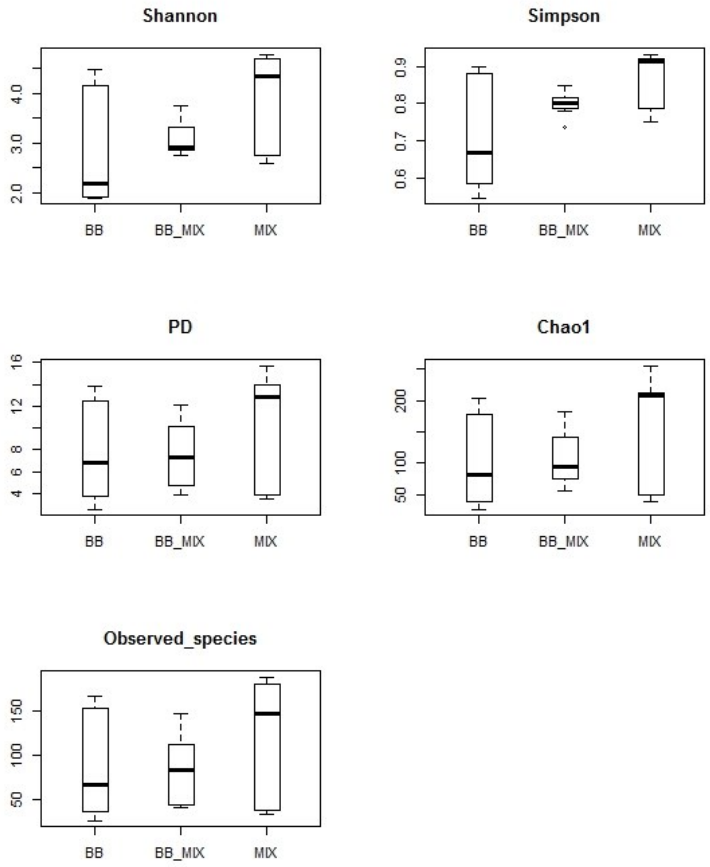




S Figure 5 Principal Coordinates Analysis (PCoA) of the faecal microbiota and MCC100 at baseline (t0 h). A: Weighted UniFrac distances; **B:** Unweighted UniFrac distances. "●" Community (COM) type microbiota; "▲" Longstay (LS) type microbiota; **red**: BB supplementation; **blue**: BB.MIX supplementation; **green**: MIX supplementation.

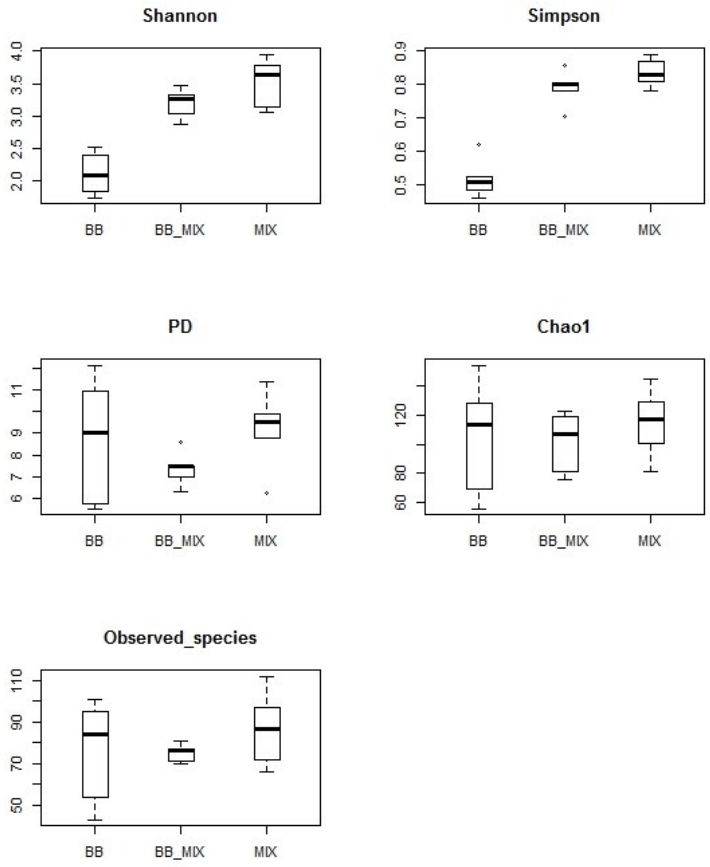
S Table 10 Alpha diversity indices for the faecal microbiotas and MCC100 at baseline (0 h).

	Alpha Diversity Index				
Microbiota	**Shannon**	**Simpson**	**PD whole tree**	**Observed species**	**Chao1**
EM278	5.5	0.9	25.3	298	387
EM425	5.5	0.9	29.6	332	404
MCC100	3.8	0.8	10.2	90	109
EM297	4.4	0.9	16.0	165	242
EM704	3.9	0.9	19.0	169	220



5634

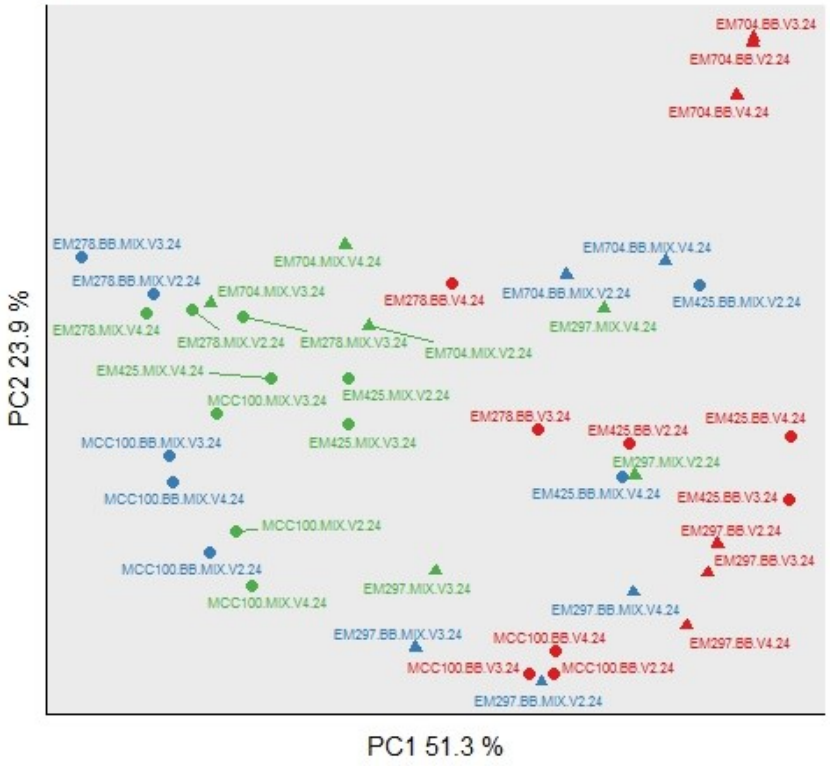
5635 **S Figure 6 Alpha diversity of the community (COM) type faecal microbiota at**
5636 **24 h.** The results refer to the aggregated faecal microbiotas EM278 and EM425. BB:
5637 blueberry supplementation; BB_MIX: blueberry and prebiotic carbohydrate mix
5638 supplementation; MIX: carbohydrate mix supplementation.



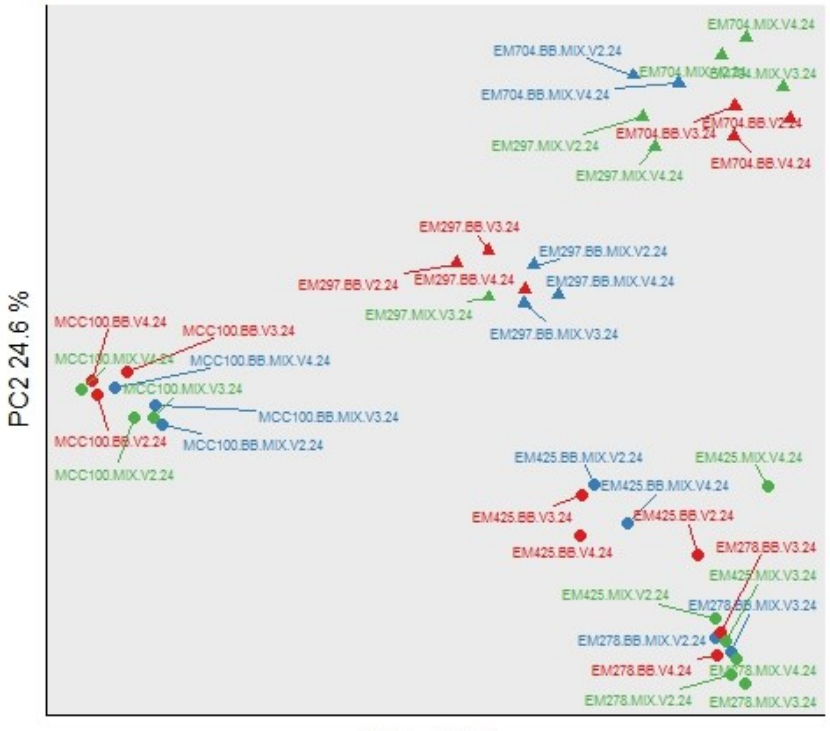
5639

5640 **S Figure 7 Alpha diversity of the longstay (LS) type faecal microbiota at 24 h.**
5641 The results refer to the aggregated faecal microbiotas EM704 and EM297. BB:
5642 blueberry supplementation; BB_MIX: blueberry and prebiotic carbohydrate mix
5643 supplementation; MIX: carbohydrate mix supplementation.

A.


EM704.BB.V3.24
EM704.BB.V2.24
EM704.BB.V4.24
EM278.BB.MIX.V3.24
EM704.MIX.V4.24
EM704.BB.MIX.V4.24
EM278.BB.MIX.V2.24
EM704.MIX.V3.24
EM278.BB.V4.24
EM704.BB.MIX.V2.24
EM425.BB.MIX.V2.24
EM278.MIX.V4.24
EM278.MIX.V2.24
EM278.MIX.V3.24
EM704.MIX.V2.24
EM297.MIX.V4.24
EM425.MIX.V4.24
EM425.MIX.V2.24
MCC100.MIX.V3.24
EM278.BB.V3.24
EM425.BB.V2.24
EM425.BB.V4.24
MCC100.BB.MIX.V3.24
EM425.MIX.V3.24
EM297.MIX.V2.24
MCC100.BB.MIX.V4.24
EM425.BB.MIX.V4.24
EM425.BB.V3.24
MCC100.MIX.V2.24
EM297.BB.V2.24
MCC100.BB.MIX.V2.24
EM297.BB.V3.24
EM297.MIX.V3.24
MCC100.MIX.V4.24
EM297.BB.MIX.V4.24
EM297.BB.MIX.V3.24
MCC100.BB.V4.24
EM297.BB.V4.24
MCC100.BB.V3.24
MCC100.BB.V2.24
EM297.BB.MIX.V2.24
PC2 23.9 %
PC1 51.3 %

B.


EM704.MIX.V4.24
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EM704.BB.V2.24
EM297.MIX.V2.24
EM704.BB.V4.24
EM297.MIX.V4.24
EM297.BB.V3.24
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EM297.BB.V2.24
EM297.BB.V4.24
EM297.BB.MIX.V4.24
EM297.MIX.V3.24
EM297.BB.MIX.V3.24
MCC100.BB.V4.24
MCC100.BB.V3.24
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EM425.MIX.V4.24
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EM425.BB.V3.24
EM425.BB.V2.24
EM425.BB.V4.24
EM278.BB.V3.24
EM425.MIX.V3.24
EM425.MIX.V2.24
EM278.BB.MIX.V3.24
EM278.BB.MIX.V2.24
EM278.BB.V4.24
EM278.MIX.V4.24
EM278.MIX.V2.24
EM278.MIX.V3.24
PC2 24.6 %
PC1 40.7 %

5644

S Figure 8 Principal Coordinates Analysis (PCoA) of the faecal microbiota and MCC100 at 24 h. A. Weighted UniFrac distances; **B.** Unweighted UniFrac distances. "●" Community (COM) type microbiota and MCC100; "▲" Longstay (LS) type microbiota; **red**: BB supplementation; **blue**: BB.MIX supplementation; **green**: MIX supplementation.

S Table 11 Development of the composition and comparison between time points of the relative abundance of orders after 24 h fermentation with community (COM) type faecal microbiota (EM278, EM425). BB: blueberry powder supplementation; BB.MIX: blueberry powder and prebiotic carbohydrate mix supplementation; MIX: prebiotic carbohydrate mix supplementation. Missing values indicate that the relevant taxon had <1% abundance in all vessels (two or three) summed for mean abundance. The mean value of the relative abundance per time point is shown.

	EM278						EM425					
OTU	**BB**		**BB.MIX**		**MIX**		**BB**		**BB.MIX**		**MIX**	
Classification	**t0**	**t24**	**t0**	**t24**	**t0**	**t24**	**t0**	**t24**	**t0**	**t24**	**t0**	**t24**
Bifidobacteriales	3.5	21.6	3.0	21.9	4.9	13.9[a]	-	-	-	-	-	-
Bacteroidales	23.3	6.3	19.4	45.5	22.3	31.8[a]	10.1	4.1	8.7	10.6	11.2	28.6
Lactobacillales	1.1	0.9	1.0	2.6	1.4	3.1[a]	0.5	32.8[a]	0.4	39.2	0.4	8.5
Clostridiales	67.0	42.9	71.5	26.8	67.6	47.1	75.6	20.5	76.9	27.3	75.0	50.5[*]
Erysipelotrichales	0.3	3.5	0.4	1.4	0.5	1.7[a]	-	-	-	-	-	-
Selenomonadales	1.0	2.2	-	-	0.4	0.6[a]	2.7	0.7[a]	2.1	0.4	1.8	0.3[a]
Firmicutes/unclassified	0.8	1.0	-	-	-	-	2.6	0.2[a]	2.7	0.02	2.7	0.6
Burkholderiales	0.8	1.1	0.9	0.5	1.0	0.7	-	-	-	-	-	-
Enterobacteriales	0.0	20.0	-	-	0.01	0.5	0.04	41.3	0.03	22.0	0.03	11.1[*]

[*] Statistically significant result, Kruskal Wallis test result $p \leq 0.05$, $p_{adj} \leq 0.05$ with Dunn's test $q_{adj} \leq 0.05$.
[a] Statistical trend, Kruskal Wallis test result $p \leq 0.05$, $p_{adj} \leq 0.1$, with Dunn's test $qa_{dj} \leq 0.1$.

S Table 12 Development of the composition and comparison between time points of the relative abundance of orders after 24 h fermentation with longstay (LS) type faecal microbiota (EM704, EM297). BB: blueberry powder supplementation; BB.MIX: blueberry powder and prebiotic carbohydrate mix supplementation; MIX: prebiotic carbohydrate mix supplementation. Missing values indicate that the relevant taxon had <1% abundance in all vessels (two or three) summed for mean abundance. The mean value of the relative abundance per time point is shown.

	EM704						EM297					
OTU	**BB**		**BB.MIX**		**MIX**		**BB**		**BB.MIX**		**MIX**	
Classification	**t0**	**t24**	**t0**	**t24**	**t0**	**t24**	**t0**	**t24**	**t0**	**t24**	**t0**	**t24**
Bifidobacteriales	-	-	-	-	-	-	2.3	0.02	-	-	-	-
Bacteroidales	10.2	3.6	10.1	13.0	10.0	30.3	15.5	1.9	16.7	18.7	19.3	14.8
Lactobacillales	0.1	72.1[a]	0.1	35.1	0.1	4.5[a]	2.5	8.6[a]	1.5	9.6[a]	4.7	28.2
Clostridiales	31.1	21.4	33.6	44.2	28.8	56.7	28.5	18.9[a]	22.1	20.2	33.1	33.2
Selenomonadales	2.3	0.3[a]	-	-	2.6	1.9[a]	0.3	1.1	0.2	1.3	-	-
Enterobacteriales	0.1	1.2	0.04	6.6	0.1	4.9	8.8	68.5[a]	6.6	44.3[a]	10.2	21.9
Synergistales	10.5	0.1	9.2	0.02	13.8	0.1[a]	23.3	0.1	21.0	0.04	7.6	0.09

[*]Statistically significant result, Kruskal-Wallis test result $p \leq 0.05$, $p_{adj} \leq 0.05$ with Dunn's test $q_{adj} \leq 0.05$.
[a] Statistical trend, Kruskal-Wallis test result $p \leq 0.05$, $p_{adj} \leq 0.1$, with Dunn's test $q_{adj} \leq 0.1$.

S Table 13 Development of the composition and comparison between time points of the relative abundance of species after 24 h fermentation with community (COM) type faecal microbiota (EM278, EM425). BB: blueberry powder supplementation; BB.MIX: blueberry powder and prebiotic carbohydrate mix supplementation; MIX: prebiotic carbohydrate mix supplementation. Missing values indicate that the relevant taxon had <1% abundance in all vessels (two or three) summed for mean abundance. The mean value of the relative abundance per time point is shown.

	EM278						EM425					
OTU	**BB**		**BB.MIX**		**MIX**		**BB**		**BB.MIX**		**MIX**	
Classification	**t0**	**t24**	**t0**	**t24**	**t0**	**t24**	**t0**	**t24**	**t0**	**t24**	**t0**	**t24**
Bifidobacterium bifidum	0.05	1.8	0.03	0.8	0.1	0.6[a]	-	-	-	-	-	-
Bifidobacterium longum	0.2	2.3	0.1	2.5	0.4	1.2	-	-	-	-	-	-
Bifidobacterium[u]	2.7	15.5	2.2	16.3	3.6	9.6[a]	-	-	-	-	-	-
Bifidobacte-riaceae[u]	0.6	2.0	0.6	2.3	0.7	2.5[a]	-	-	-	-	-	-
Bacteroides caccae	0.9	0.4	0.7	3.2	0.8	5.7[a]	-	-	-	-	-	-
Bacteroides dorei	7.5	0.7	6.4	0.4	7.2	1.4[a]	-	-	-	-	-	-
Bacteroides eggerthii	1.9	0.2	1.7	0.8	2.0	0.5[a]	-	-	-	-	-	-
Bacteroides fragilis	-	-	-	-	-	-	0.8	0.6	0.6	10.1	0.8	15.2
Bacteroides thetaiotaomicron	0.6	0.3	-	-	0.4	0.4	-	-	-	-	0.1	1.1[a]
Bacteroides uniformis	7.8	2.1	6.7	38.3	7.4	19.2[a]	4.5	2.5	3.6	0.1	5.5	8.2
Bacteroides[u]	-	-	-	-	0.5	1.8	-	-	-	-	0.1	1.1[a]
Alistipes[u]	1.1	1.3	-	-	1.2	1.4	-	-	-	-	-	-

OTU	EM278						EM425					
	BB		BB.MIX		MIX		BB		BB.MIX		MIX	
Classification	t0	t24	t0	t24	t0	t24	t0	t24	t0	t24	t0	t24
Streptococcus[u]	1.1	0.9	1.0	2.6	1.3	3.1	0.4	32.1[a]	0.2	39.1	0.2	8.5
Clostridium sensu stricto[u]	-	-	-	-	-	0.5	7.8	5.0[a]	0.6	5.7	0.4	12.3[a]
Anaerostipes hadrus	2.3	11.2	2.6	11.3	2.5	7.8	-	-	0.7	6.7	0.8	8.1
Blautia faecis	2.3	0.5	2.7	2.0	2.0	4.3	-	-	-	-	-	-
Blautia luti	2.0	3.0	2.4	0.7	2.2	2.3	-	-	-	-	-	-
Blautia[u]	4.5	1.9	5.9	0.9	4.2	2.1	2.6	0.1	2.8	1.6	2.4	3.4
Clostridium XlVa[u]	-	-	-	-	0.1	0.8[a]	0.1	0.5	-	-	-	-
Coprococcus catus	-	-	0.3	1.2	-	-	0.2	1.1[a]	0.2	0.8	-	-
Coprococcus comes	0.6	1.3	-	-	0.8	4.3[a]	0.2	0.6	0.2	0.9	-	-
Eubacterium rectale	15.1	0.5	16.3	2.7	16.6	10.2	-	-	-	-	-	-
Lachnospiraceae[u]	13.6	5.8	15.4	5.2	11.9	9.8[a]	35.4	3.7[a]	34.3	1.4	38.3	12.0[a]
Clostridium XI[u]	-	-	-	-	-	-	0.2	1.4	0.1	0.1	-	-
Faecalibacterium prausnitzii	9.9	12.7	8.8	0.2	10.1	1.4	7.9	2.5	8.8	0.1	7.9	5.3[a]
Gemmiger formicilis	-	-	-	-	1.4	0.1	2.0	0.1	2.7	7.4	1.8	2.0
Ruminococcaceae[u]	2.2	1.7	2.3	0.2	2.1	0.5[a]	7.6	0.2	7.8	0.1	6.2	0.7[a]
Clostridiales[u]	2.7	0.1	2.5	0.1	2.5	0.2[a]	3.8	0.2[a]	4.0	0.1	3.6	0.2
Clostridium XVIII[u]	0.1	3.2	0.2	1.3	0.3	1.4	-	-	-	-	-	-

	EM278						EM425					
OTU	**BB**		**BB.MIX**		**MIX**		**BB**		**BB.MIX**		**MIX**	
Classification	**t0**	**t24**	**t0**	**t24**	**t0**	**t24**	**t0**	**t24**	**t0**	**t24**	**t0**	**t24**
Phascolarctobacterium faecium	0.4	2.0	-	-	0.2	0.6[a]	-	-	-	-	-	-
Firmicutes[u]	0.8	1.0	-	-	-	-	2.6	0.2[a]	2.7	0.02	2.7	0.6[a]
Shigella[u]	0.02	9.3	-	-	-	-	0.04	40.8	0.03	21.8	0.03	11.1[a]

[*]Statistically significant result, Kruskal-Wallis test result $p \leq 0.05$, $p_{adj} \leq 0.05$ with Dunn's test $q_{adj} \leq 0.05$
[a]Statistical trend, Kruskal-Wallis test result $p \leq 0.05$, $p_{adj} \leq 0.1$, with Dunn's test $q_{adj} \leq 0.1$
[u]Unclassified: not classified at a finer phylogenetic level than the indicated

5672

S Table 14 Development of the composition and comparison between time points of the relative abundance of species after 24 h fermentation with longstay (LS) type faecal microbiota (EM704, EM297). BB: blueberry powder supplementation; BB.MIX: blueberry powder and prebiotic carbohydrate mix supplementation; MIX: prebiotic carbohydrate mix supplementation. Missing values indicate that the relevant taxon had <1% abundance in all vessels (two or three) summed for mean abundance. The mean value of the relative abundance per time point is shown.

	EM704						EM297					
OTU	**BB**		**BB.MIX**		**MIX**		**BB**		**BB.MIX**		**MIX**	
Classification	**t0**	**t24**	**t0**	**t24**	**t0**	**t24**	**t0**	**t24**	**t0**	**t24**	**t0**	**t24**
Bifidobacterium[u]	-	-	-	-	-	-	1.6	0.01	-	-	-	-
Bacteroides caccae	-	-	-	-	0.4	1.4	-	-	-	-	-	-
Bacteroides cellulosilyticus	-	-	-	-	0.6	1.3	-	-	-	-	-	-
Bacteroides dorei	1.5	0.4[a]	1.0	0.7	1.1	0.9	4.0	0.2[a]	4.8	0.7[a]	4.1	1.1
Bacteroides fragilis							7.5	0.3[a]	7.4	8.8	8.9	8.5
Bacteroides thetaiotaomicron	3.3	2.8	4.4	11.9	3.3	25.8	0.3	1.1	0.4	5.1	0.5	2.7
Bacteroides[u]	-	-	-	-	-	-	0.8	0.2	0.9	3.9	1.2	2.4
Methanobrevibacter smithii	10.4	0.04	-	-	7.5	0.03[a]	-	-	-	-	20.7	0.003
Enterococcus[u]	-	-	-	-	-	-	1.1	8.6[a]	0.5	9.6[a]	1.6	28.1
Streptococcus[u]	0.1	72.0[a]	0.1	35.0	0.1	4.5[a]	-	-	-	-	-	-
Clostridium baratii	-	-	0.7	2.8	0.7	1.7[a]	-	-	-	-	-	-
Clostridium disporicum	9.4	0.8	10.3	4.6	8.4	4.4	-	-	-	-	-	-

OTU	EM704						EM297					
	BB		BB.MIX		MIX		BB		BB.MIX		MIX	
Classification	**t0**	**t24**	**t0**	**t24**	**t0**	**t24**	**t0**	**t24**	**t0**	**t24**	**t0**	**t24**
Clostridium sensu stricto[u]	0.1	6.9	0.1	10.3	0.1	25.5	-	-	0.003	0.8[a]	-	-
Blautia[u]	0.2	1.7[a]	0.1	2.1	0.1	5.0[a]	0.2	1.0	0.2	1.1	0.2	2.7
Clostridium hathewayi	-	-	-	-	-	-	1.6	2.4	1.2	7.0	1.9	4.7
Clostridium XlVa[u]	-	-	-	-	0.2	1.4[a]	2.7	0.8	2.3	0.9[a]	3.4	1.6
Ruminococcus torques	1.4	0.1	1.4	0.05	1.2	0.3[a]	6.1	0.1[a]	4.7	2.4	7.0	0.03
Eubacterium eligens	-	-	-	-	-	-	0.1	0.5	0.1	3.0	-	-
Lachnospiraceae[u]	1.4	0.1[a]	1.4	0.03	-	-	2.5	2.3	2.0	3.6[a]	3.5	3.1
Clostridium glycolicum	0.1	1.4	0.1	3.5	-	-	-	-	-	-	-	-
Clostridium XI[u]	0.2	1.9[a]	0.2	11.0	0.2	3.9[a]	-	-	-	-	-	-
Ruminococcaceae[u]	2.7	1.5[a]	2.5	4.0	2.9	5.1[a]	5.4	0.02	4.7	0.03	5.4	0.02
Clostridiales[u]	1.8	0.1	2.1	0.1	1.9	0.6[a]	2.0	0.5[a]	1.5	0.5[a]	2.3	1.4
Phascolarctobacterium faecium	2.3	0.3[a]	-	-	2.6	1.9[a]	0.3	1.1	0.2	1.3	-	-
Escherichia/Shigella[u]	0.01	0.9	0.01	6.5	0.02	4.8	7.0	66.8[a]	5.4	44.3[a]	9.0	20.3
Klebsiella[u]	-	-	-	-	-	-	1.8	1.6	-	-	1.2	1.1
Cloacibacillus evryensis	10.3	0.1	9.0	0.02	13.7	0.1[a]	23.2	0.1	20.9	0.04	7.6	0.1
Akkermansia muciniphila	30.2	0.03	-	-	34.7	0.2	-	-	-	-	-	-

[*]Statistically significant result, Kruskal-Wallis test result $p \leq 0.05$, $p_{adj} \leq 0.05$ with Dunn's test $q_{adj} \leq 0.05$
[a]Statistical trend, Kruskal-Wallis test result $p \leq 0.05$, $p_{adj} \leq 0.1$, with Dunn's test $q_{adj} \leq 0.1$
[u]Unclassified: not classified at a finer phylogenetic level than the indicated

Chapter 5

General Discussion

Considerations for advancing gut microbiota modulation research for older people

The population over 60 yrs old is growing fast and people over 80 yrs are expected to comprise 13% of the European population by 2080 (http://ec.europa.eu/eurostat/statistics-explained/index.php/Population_structure_and_ageing). The extension of human life expectancy comes with increased risk of age-related conditions such as cognitive decline and non-communicable disease (metabolic disease, CVD, cancer), which places national economies and health care systems under significant stress (Divo *et al*., 2016). While habitual diet can be the major source of key nutrients that could prevent or at least, ameliorate age-related conditions, the high prevalence of malnutrition in both community- and long term care unit- dwelling older people has increased the need for innovative nutritional recommendations and strategies, and the design of novel functional food products targeting the specific nutritional needs of the elderly population (de Groot *et al*., 2016; Hill *et al*., 2016; Moore *et al*., 2018).

There is an increasing effort to develop general or targeted gut microbiota therapeutics for the older population, similar to the efforts aimed at diseases in which the associated microbiota dysbiosis could be treated with prebiotics, probiotics or bacteriotherapy (reviewed in Chapter 1; Salazar *et al*., 2017). While Salazar *et al*. (2017) in their recent review pointed out that more intervention studies on older population using a wide range of probiotics and prebiotics are needed, I have also emphasised in Chapter 1.2 the fact that there is a scarcity of studies using faecal microbiota from older subjects (≥65 yrs) in *in vitro* and also in *in vivo* preclinical experiments. Furthermore, there is a lack of preclinical trials using microbiota from non-healthy older subjects (reviewed in Chapter 1.2).

In vitro screening of the "elderly" gut microbiota against an array of potential microbiota modulators (e.g. prebiotics, probiotics, LBT), could be an important stepping stone to advance the preclinical research of microbiota therapeutics targeting the older population. Outcomes of *in vitro* screens can lead to the design of *in vivo* preclinical trials and subsequently, to human interventions (O'Toole *et al*., 2017; Schmidt *et al*., 2018). In this research thesis, faecal microbiota from healthy and non-healthy (frail) older subjects were used in the preclinical trials described in Chapters 2, 3 and 4. The use of both "healthy" and "non-healthy" microbiota from elderly people reflects the current scientific interest to develop both preventive and therapeutic approaches for gut microbiota modulation (Derrien and Veiga, 2016).

The choice between aiming at a general or a more precise gut microbiota modulation depends on the context of what is considered the healthy or normal state of the microbiota, and whether whole community or specific taxon perturbations are associated with the non-healthy state of interest. Targeted and untargeted microbiota modulations can be achieved through the use of pre/pro-biotics and LBT, or through FMT (reviewed in Chapter 1.2; Schmidt *et al*., 2018). Importantly, under the current updated concept of prebiotics, (potential) prebiotics can serve in untargeted microbiota modulation approaches because retention of the phylogenetic diversity of the whole microbiota community in combination with increased SCFA production especially butyrate, is emerging as a desirable outcome of prebiotic administration (reviewed in Chapter 1.2; Gibson *et al*., 2017).

Examples of precision approaches targeting a certain microbiota component include the following: *F. prausnitzii* is proposed as a therapeutic target for Crohn's disease (Sokol *et al*., 2008; Pascal *et al*., 2017), therefore, the taxon could be supplemented

in patients or targeted with prebiotics. The discovery of CRC biomarker bacterial clusters (Flemer *et al*., 2017) may call for a targeted approach that will aim at a wide but defined portion of the microbiota community of patients. Importantly, for the rational design of precision gut microbiota therapeutics, some degree of causation beyond mere associations, between biomarker taxa and disease needs to be established before aiming at specific microbiota interventions (Schmidt *et al*., 2018).

It is also important not to overstate the beneficial effects of certain gut bacteria or "incriminate" others for deleterious effects based solely on association studies and without placing the effect in an ecological context (e.g. diet, immunity, cross-talk with other microbiota members), as the examples of the LBT based on the "beneficial" taxon *A. muciniphila* and *P. copri* have shown (Cani, 2018; Cirstea *et al*., 2018). Another example is the normalisation of dysbiosis and inflammation by precision tailoring of the expanded Enterobacteriaceae population during the inflamed stage of colitis in mice (Zhu *et al*., 2018). This potentially pathobiont taxon which is a common member of the gut microbiota was not eradicated but its abundance was restored to pre-inflammation levels.

While some conditions may require the targeting of certain taxa or clusters, others will require a general effect on the whole microbiota community. For example, the association of metabolic disease with low diversity and low gene counts in the gut microbiota (Le Chatelier *et al*., 2013) indicates the need for general modulations, i.e. to target of the whole community for the increase of the phylogenetic diversity, in order to decrease the risk for metabolic conditions. Interestingly, boost of the phylogenetic diversity of the gut microbiota of "westernised/industrialised" cohorts in which decreased phylogenetic diversity is a potential risk factor for various

"modern" diseases such as metabolic disease and IBD, could be key for general approaches towards maintaining health and lowering the risk of disease in the general population (Moeller *et al*., 2017).

Identifying the "healthy adult" microbiota has been challenging (Lloyd-Price *et al*., 2016). A "functional healthy core" in which different microbial lineages confer a similar metabolic activity due to metabolic redundancy in the gut microbiota has been proposed as a more relevant notion compared to a core of taxa for which consensus across cohorts is often prevented by inter-individual compositional variations (Lloyd-Price *et al*., 2016). Compositional patterns, i.e. enterotypes (discussed in Chapter 1.1), in spite of the controversy with which the concept was initially met, may offer a useful way to stratify "healthy" microbiota compositional profiles across subjects (Costea *et al*., 2018).

In depth understanding of microbiota assembly dynamics can be key to general microbiota modulatory strategies. In spite of inter-individual species/strain level differences, and especially for "healthy" microbiomes, these dynamics are most likely governed by "universality" reflected in the pathways of inter-species cross-talk, resilience to stressors and in the combination of compositional adaptiveness and longitudinal robustness (Bashan *et al*., 2016; Gilbert *et al*., 2018). Dysbiotic microbiota dynamics are more difficult to predict because this "universality" may be interrupted and some authors argue for "stochastic" changes in the dysbiotic microbiota (Bashan *et al*., 2016; Zaneveld *et al*., 2017).

The wide distance observed in β-diversity analysis among patients (e.g. Crohn's disease) compared to the close clustering of healthy controls, indicates that the underlying dysbiosis may in some conditions, lead to an unstable microbiota state

and unpredictable (stochastic) shifts from the healthy microbiota profile (Zaneveld *et al*., 2017). The deregulated dynamics of the "dysbiotic microbiota" which is most likely characterised by unpredictable responsiveness to stressors, may also be hampering responsiveness to therapeutics. In depth profiling of the gut microbiota compositional changes in "perturb-to-predict" experiments (e.g. in *in vitro* and *in vivo* colon models), offer valuable insight to microbiota dynamics because they can help typify shifts of the microbiota from baseline as being stochastic or reproducible as a response to the experimentally imposed challenge (e.g. response to diet) (Zaneveld *et al*., 2017; Schmidt *et al*., 2018).

The re-evaluated concept of the enterotypes in the analysis of microbiota composition could further refine the identification of predictable and stochastic microbiota responses (Costea *et al*., 2108). Furthermore, many studies have reported responders and non-responders to prebiotic intervention, underlying the importance of the baseline microbiota composition in "perturb-to-predict" approaches (Duncan and Flint, 2013; Zhao *et al*., 2018). To date, *Bacteroides* and *Prevotella* have been identified as key taxa that define major enterotype profiles among individuals, and responsiveness variations, for example as demonstrated in an *in vitro* colon system after dietary fibre supplementation (Chen *et al*., 2017; Costea *et al*., 2018).

Taken together, efforts to categorise microbiota responses to extrinsic challenges (e.g. diet, antibiotics, LBT, pathogens) in predictable or stochastic manners, overcoming the inter-individual variations by stratifying responses according to enterotypes and baseline microbiota characteristics, may facilitate generalised and at the same time precision approaches in health-maintenance and therapeutic strategies, overcoming the need for personalised therapeutics (Sonnenburg and Backhed, 2016).

In order to extend our definition of the "healthy" microbiota, an interesting question to ask is how different is the responsiveness of the "healthy older" from the "healthy younger" gut microbiota to perturbations. The "healthy older" may not significantly differ in composition from the "healthy younger adult" microbiota as observed in principal coordinates (PCoA) analysis in various studies (Claesson *et al*., 2012; Bian *et al*., 2017). In the *in vitro* experiments described in Chapters 2 and 4, microbiota from younger adults was not used. However, it would be interesting to examine in future preclinical *in vitro* studies the comparative responsiveness of healthy "young" and "old" microbiota to substrate supplementation/perturbation, both in taxa and SCFA production.

In vitro microbiota perturbation tests (e.g. challenge of the microbiota with candidate pre/pro-biotic substrate, LBT, pathobionts), in which simulation of the host factors that affect the microbiota composition (e.g. lower immune regulation in the elderly; Kundu *et al*., 2017) cannot be achieved, apart perhaps from the slower retention time in the elderly colon (e.g. in Fehlaub *et al*., 2015), may allow for a better understanding of the "intrinsic" potential of the "young" and "old" healthy microbiota to respond to challenges. The potential differences could be associated to different strains being differentially enriched in the two types of microbiota (Zhu *et al*., 2015). Strain level differences between microbiotas are often underestimated because it is difficult to discern them based on phylogenetic gene markers such as 16S rRNA gene (Zhu *et al*., 2015); however, strain level analysis will refine our understanding of the microbiota dynamics and functionality. Identification of such fine strain-level differences may improve the outcome of gut microbiota modulation strategies (De Filippis *et al*., 2016) and open a window for preventive modulations targeting the progression of ageing in the microbiota.

The use of synthetic minimal microbial communities (reviewed in Chapter 1.2,; experiments in Chapter 4), can be a valuable tool for studying how key microbiota members respond to dietary substrates, and especially in *in vitro* systems, and they offer the potential to study the microbiota down to strain level properties. Rationally assembled artificial microbiota communities that are designed to resemble ”healthy” or “dysbiotic” gut microbiota or microbiotas of certain enterotypes, will allow for controlled and reproducible experiments through which associations, function, metabolism, cross-feeding and other parameters can be studied in a reduced in complexity ecosystem (de Roy *et al*., 2013). Importantly, *in vitro* studies employing artificial microbiota communities can be the basis for the design of LBT intervention in which microbiota consortia and dietary context (i.e. substrates that can sustain the viability and diversity of the consortium) can be matched in order to achieve the desired response (e.g. increased butyrate production by increasing the fibrolytic bacteria in the colon) in microbiotas that otherwise may fail to respond due to missing responsive members (Makki *et al*., 2018).

The reproducibility within studies and the comparability across studies that can now be achieved by thorough documentation of protocols from the wet lab to the data analysis, can maximise the information extracted from microbiome studies, leading to improved translatability and rationally designed clinical applications (Knight *et al*., 2018). While next generation 16S rRNA amplicon sequencing has been a major analysis method in the microbiota studies to date, it is apparent that in order to refine the microbiota taxonomic and functionality analysis, a combination of –omics methods should be employed (e.g. metagenomics, metatranscriptomics) when economic resources, sample availability and time constrains allow for it (Knight *et al.,* 2018). Especially the metagenomics approach, allows for high taxonomic

resolution of both the bacterial component of the microbiota community and the whole microbial component, i.e. fungi, yeasts, archaea and viruses and phages, which although underrepresented in most microbiota studies, still impacts health and disease (Cani, 2018). Furthermore, accurate profiling of the microbiota composition could benefit from a combination of qualitative (16S rRNA gene amplicon sequencing) and quantitative methods (e.g. quantitative PCR) in order to detect true changes in cell counts in combination to relative abundance changes (Vandeputte *et al*., 2017; Knight *et al*., 2018).

Any prediction that is generated in *in vitro* studies requires validation in *in vivo* systems and ultimately in "real life" clinical trials, before scientific advances in the field of gut microbiota research can be responsibly disseminated to the public (Cani, 2018; Schmidt *et al*., 2018). Importantly, some advances in gut microbiota research are already reaching the public and health authorities are promoting science-backed dietary recommendations (Derrien and Veiga, 2016). Dietary recommendations represent low-cost health-improving strategies but require careful dissemination strategies from the laboratories to society and adherence by the public. Cohort studies show that adherence to international and national dietary guidelines may significantly improve public health (Batis *et al*., 2016; Biesbroek *et al*., 2017) and even increase the life expectancy of the older population (Jankovic *et al*., 2014; Jankovic *et al*., 2015).

In spite of the importance of dietary guidelines, adherence is often challenging. Functional foods supplemented with next generation prebiotics and/or probiotics or microbial products potentially regulated as food products (Derrien and Veiga, 2016;

Brodman *et al*., 2017) are expected to play a significant role in the amelioration of health across all ages and in particularly in the elderly population.

Widely used plant- and animal- derived dietary substrates as a source for novel prebiotics

Plants (fruits, vegetables, legumes) are the main source of substrates with potential prebiotic activity, e.g. dietary fibre (reviewed in. Makki *et al*., 2018) and in the search for new prebiotics, dietary substrates of plant origin are being investigated. There is increasing interest in the study of the effect of dietary polyphenols in the gut microbiota, found in fruits, vegetables and legumes (Derrien and Veiga, 2016). The health relevance of polyphenol consumption is associated with the gut microbiota metabolism of unabsorbed polyphenols and the subsequent release of bioactive phenolic compounds (Marchesi *et al*., 2015). Furthermore, some recent studies have focused on the cumulative prebiotic effect of polyphenols and dietary fibre, because apart from the documented health benefits of these two food components, the binding of polyphenols to dietary fibre (e.g. the case of plant cell wall) may increase the bioavailability of the polyphenols in the distal colon (discussed in Chapter 4).

Another less investigated source of potentially prebiotic substrates is milk. As reviewed in Chapter 1.3, milk and its carbohydrate components are drawing scientific attention due to BMOs having complex structures similar to HMOs. The structural complexity of HMOs is associated to the HMOs prebiotic activity (reviewed in Chapter 1.2). A similar to HMOs structural complexity has not been identified in the plant derived prebiotic candidates investigated to date (Chapter 1.2). The large-scale isolation (or production) of HMOs has been challenging and therefore, there are limitations in the use of HMOs for study purposes or for food

supplementation (Bode *et al*., 2016). Should prebiotic activity of BMOs be documented (as recent studies are indicating; see Chapters 1.3 and 3), they could replace HMOs in various large-scale *in vitro*, *in vivo* and importantly, in clinical studies, thus, extending our knowledge on the effect of these non-plant derived OS on the gut microbiota.

The studies on the effect of isolated milk components such as BMOs or GMP (Chapter 2) on the gut microbiota can reveal new opportunities for functional foods design (https://www.nutrition.org.uk/nutritionscience/foodfacts/functional-foods.html?limitstart=0) and give new perspectives for the industrial and commercial exploitation of dairy industry residual by-products. Isolation of BMOs, and GMP, from whey concentrates may present a productive way for the dairy industry to resolve the challenging whey surplus problem while also extracting valuable compounds for foods supplementation (Oliveira *et al*., 2015; Bode *et al*., 2016).

To my knowledge (reviewed in Chapter 1.3), research has yet to thoroughly assess the effect of regular milk consumption on the microbiota composition, an area of study which could complement the array of health benefits known for milk consumption. Evidence on the prebiotic potential of (whole, unfermented) milk, such as those presented in Chapter 3, in combination with large cohort data and meta-analysis (reviewed in Chapter 1) showing that milk consumption does not correlate with detrimental health effects, would add compelling scientific evidence towards milk inclusion in a balanced diet. The inclusion of milk in the diet of the elderly population is of particular relevance because milk is a source of the vitamins B and D among others, and of protein and micronutrients such as calcium, the dietary

5920 deficiency of which is associated with degenerative disease in older age (de Groot,
5921 2016).

5922 **Research thesis contribution to current literature and future work**

5923 In this research thesis I investigated the modulatory effect on the human gut
5924 microbiota of selected food ingredients, i.e. milk and milk-derived GMP, and berry
5925 fruit, focusing on older subjects. The aim was to provide scientific evidence for the
5926 prebiotic potential of these products and therefore, evidence to warrant the design of
5927 informed future clinical trials in order to establish the effect of the selected products
5928 on GIT health.

5929 In Chapter 1.3, I have reviewed the existing evidence on the potential of the selected
5930 dairy food ingredients to be used in approaches to modulate the gut microbiota; I
5931 have also reviewed the existing knowledge on the health benefits associated with the
5932 consumption of these products. In Chapter 1.2, I reviewed the major experimental
5933 models that can be used in the study of gut microbiota modulation. Chapter 2
5934 provides experimental evidence for the potential of GMP to modulate *in vitro* the gut
5935 microbiota of older subjects by promoting the growth of several health-relevant taxa
5936 and by sustaining the microbiota diversity close to baseline starting point. As I
5937 reviewed in Chapter 1.3, previous *in vitro* studies yielded controversial evidence on
5938 the prebiotic potential of GMP. One possible reason for this is the fact that they did
5939 not examine the whole microbiota community changes that GMP can promote, but
5940 rather they focused on identifying changes in specific taxa such as *Lactobacillus* and
5941 *Bifidobacterium*, measured by quantitative molecular methods such as FISH (Bruck
5942 *et al*., 2002; Hernandez-Hernndez *et al*., 2011). In Chapter 2, I profiled the changes

in the whole community and identified affected taxa beyond the "traditional" targets of prebiotics.

Importantly, in the *in vitro* experiments I used faecal samples from subjects in which low phylogenetic diversity correlated with poor health and frailty. As reviewed in Chapter 1, frailty is associated to dysbiosis, it is prevalent in the elderly population and it is a risk factor for morbidity. Apart from examining the potential of GMP to sustain the phylogenetic diversity of the "frail" microbiota, we exposed a major limitation in the use of low-diversity faecal samples in *in vitro* models. Reduced diversity can lead to reduced responsiveness of the microbiota, translating into fewer competent taxa being overrepresented in the compositional profile after fermentation, as seen in Chapter 2 figures.

This observation could be reflecting "real life" observations from other studies: low richness faecal microbiota (measured by alpha diversity, species diversity indexes and gene counts) in obese subjects, was not only predictive of disease but also of low responsiveness to dietary intervention (Cotillard *et al*., 2013; Le Chatelier *et al*., 2013). Some restoration of microbiota dysbiosis was achieved by dietary intervention but the underlying low-grade inflammation was not significantly ameliorated (Cotillard *et al*., 2013), implying that low species richness leads to low metabolic potential (e.g. SCFA production) through limited functional redundancy in the microbiota.

In spite of the limitations, *in vitro* colon models can be a valuable tool for the study of the modulation of the "frail" microbiota. One improvement for future studies is the use of a rich basal medium which can be supplemented with the test substrate (similar to the one used in Chapter 4). The adaptation of the faecal microbiota in *in*

vitro systems comes with the reduction of the alpha diversity (discussed in Chapters 1.2 and 2); a rich basal medium may be necessary when using microbiota with reduced diversity in order to sustain the maximum portion of the baseline diversity and therefore, its dynamics and functionality.

In Chapter 2, I discussed the choice not to pool the faecal samples that were used. An interesting adjustment in the study design for future experiments, would be the pooling of "frail" microbiotas following the rationale of Zaneveld *et al*. (2017). The comparative analysis of the responsiveness of the pooled "frail" faecal microbiota against single-donor "frail" microbiotas could reveal whether the observed changes are reproducible or stochastic because in the pooled microbiota the stochastic responses may be minimised (Zaneveld *et al*. 2017).

In Chapter 3, I described the preclinical trial where the potential of milk and GMP to modulate the gut microbiota was examined in an *in vivo* colon model of humanised mice. With the preclinical trial we aimed at evaluating the findings of the *in vitro* trial with regard to GMP prebiotic activity, and at performing an in-depth profiling of the changes that milk confers on the gut microbiota and thus, to provide evidence for health benefits of milk consumption through its potential prebiotic activity.

In the mouse model used here, we found that lactose free milk was the most efficient in retaining gut microbiota diversity equally to or followed by soy-protein control diet. Although whole milk retained higher microbiota diversity compared to GMP, no significant differences were detected between the two feeding regimes. Importantly, both milk diets positively affected the relative abundance of health-relevant taxa such as *R. bromii*, in a gender-dependent manner. Same gender mice are often used in microbiome studies in order to obtain more homogenous results.

However, it is important to address gender variations not only in murine studies (Wang *et al*., 2016; Fransen *et al*., 2017) but also in human studies in order to identify the extent of the impact of the endocrine system on the gut microbiota inter-individual variation and therefore, illuminate another aspect of the gut microbiota dynamics (Neuman *et al*., 2015).

The inherent limitations of the mouse model used in Chapter 3 experiments (analysed in Chapters 1.2, 3), generated some questions that may be reducing the translatability of the results. For example, the murine gut favours the establishment and prevalence of xenomicrobiota Bacteroidetes including Bacteroidaceae and Porphyromonadaceae, whereas it is refractory to Firmicutes / Lachnospiraceae xenomicrobiota colonisation (reviewed in Chapter 1.2). Glycomacropeptide contains a high portion of sialic acid that, as discussed in Chapter 3, may promote the growth of Bacteroidetes taxa. Therefore, testing a potentially Bacteroidetes promoting substrate in a gut microbiota model that is "biased" towards Bacteroidetes enrichment, may not have been the most useful approach for identifying the prebiotic potential of GMP. Importantly, it was the milk diets that led to the smallest Bacteroidetes/Firmicutes ratio, tipping the scale towards an increased Firmicutes relative abundance. However, because of the poor Lachnospiraceae colonisation (e.g. Figures 18 and 21) we could not identify effects on a wide range of Lachnospiraceae taxa.

It has been proposed that for the investigation of the effect of diet on the gut microbiota, the use of conventional mice with their intact indigenous gut microbiota and full-range taxonomic representation may be a more informative approach compared to conventionalisation or humanisation (Arrieta *et al*., 2016). In spite of

limitations of the study model, the promising findings of our pre-clinical trial coupled with the fact that most of the cohort studies indicate the health benefits of moderate milk consumption, warrant a human trial searching for microbiota effects.

Importantly, mice like other rodents, may not fully tolerate lactose (de Heijning *et al*., 2015) and it can be inferred that the differential effect of the two types of milk on the gut microbiota described in Chapter 3, is due to the presence or absence of residual lactose reaching the colon. In LP humans, lactose is fully digested and absorbed in the small intestine. Whether or not this means that lactose free milk and whole milk have similar gut microbiota modulatory effect in LP humans, remains to be investigated in a clinical trial. Furthermore, because LNP subjects can tolerate an average of 1 glass of whole milk per day, it would be also interesting to further investigate the prebiotic potential of whole milk in LNP subjects.

Another interesting question in the search of the effect of milk on the gut microbiota is if different lactose free milk preparations have the same effect on the human gut microbiota composition. The lactose-free milk used in Chapter 3 was prepared after lactose was filtered and the remaining lactose was hydrolysed (https://www.valio.com/articles/lactose-intolerance-is-real-and-common/). Other preparations involve only lactose hydrolysation to glucose and galactose. Potentially, low load of simple sugar (e.g. the case of filtered lactose free milk preparations) reaching the small intestine microbiota that competes with the human host for the uptake of these compounds, can affect the downstream microbiota dynamics differentially to higher simple sugar load (Zoetendal *et al*., 2012; Marinez-Guryn *et al*., 2018).

In Chapter 4, I described the exploratory experiments towards identifying the *in vitro* effect of the blueberry powder on the gut microbiota of elderly subjects and the bacterial consortium MCC100. We hypothesised that the high sugar content (e.g. fructose) of the blueberry powder, resulted in enrichment of the microbiota in streptococci as these organisms are known to be fast utilisers of simple sugars (Zoetendal *et al*., 2018). Only the presence of bifidobacteria outcompeted streptococci in the utilisation of the provided substrates, showing the importance of the baseline microbiota in the responsiveness to the dietary substrates. Interestingly, bifidobacteria taxa were present at baseline in one of the two LS microbiotas used; however, they failed to respond to supplementation similarly to what observed when present in the COM type microbiota. If this is an indication of deregulated microbiota "universality" remains to be further examined in future experiments *in vitro* experiments using both faecal and artificial microbiotas.

A multi-stage fermenter involving compartmentalisation of interconnected vessels with different pH mimicking parts of the GIT might have allowed for fastidiously growing fibre utilisers to grow; under *in vivo* conditions, simple sugars would have been digested or absorbed in the upper parts of the GIT. Results of the phenolic compound chemical analysis are awaited. In future experiments that will complete this exploratory work, isolated phenolic compounds will be used in *in vitro* fermentation experiments in order to identify specific microbiota changes caused by selected phenolic compound supplementation free of complicating sugars being present.

The artificial consortium was assembled by our laboratory in order to mimic the faecal microbiota of a healthy adult. The 100 selected strains belong to the laboratory

microbiome collection of faecal bacteria isolated from healthy donors; the isolation work was part of this PhD research. A manuscript with details on consortium assembly and relevant further work is in preparation as mentioned in Chapter 4 (Materials and Methods). As discussed before (Chapters 1, 5), the use of minimal synthetic microbiotas is expected to facilitate diet-microbiota research. To my knowledge, this is a first attempt to use a minimal "healthy" faecal microbiota in the study of the effect of diet on the microbiota. Future experiments with isolated phenolic compounds and potentially with consortia that will mimic microbiotas of different enterotypes are expected to elucidate the role and associations of various gut microbiota members in the metabolism of dietary polyphenols and other dietary components with potential prebiotic activity.

5.1 References

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